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AUTHOR(S):

Matsumura, Kazuaki

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KAZUAKI MATSUMURA

2004

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Kyoto University

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## General introduction

Biomedical materials have been widely applied in the clinical surgery, for example, pacemakers as artificial organs [1,2], suture [3] and hypodermic syringes [4] as disposable materials, carriers of drug delivery systems [5,6] and so on. Among these, it is said that the oldest artificial materials applied to a body were dental implants, which were used in ancient Roman world from first or second century AD [7]. From these ages to 19th century, dental implants have been applied to only noble people and almost no marked improvement has been seen, because the treatment of the lost tooth doesn't lead to a fatal result and was a kind of luxury. However, the surgery in these days is aimed to not only save patients but also enhance the quality of life (QOL) of them. In dental area, lost teeth were generally covered by dentures. However, dentures sometimes cause an alveolar bone resorption and lead to loss of the fitness because the bone is remodeled by the biting force and then QOL declined. Dental implants are surgically placed into the jawbone simulating the anchoring of a tooth by its root, and much attention has been paid for the dental implants in latter part of 20th century. Bränemark et al. developed the osseointegrated implant in 1969 [8]. Osseointegration means that a direct structural and functional connection between a bone and the surface of an implant. After the invention, lots of osseintegrated implants were using all over the world [9-14]. Currently, the osseointegrated type implant is almost acceptable because it can be tightly fixed with an alveolar bone [15]. However, the direct impact of occlusal and bruxing forces has often caused the absorption of the alveolar bone and loosening of the implant because of the mechanical disharmony between the natural teeth and the implant [16,17]. In contrast, a natural tooth root is covered with a periodontal ligament (PDL), mainly consisting of active fibroblasts and collagen fibers [18,19], and plays

quite an important role as a shock absorber [20] against the mastication impact and as a receptor of forces. PDL lies between the cementum and the alveolar bone and these three components are called periodontium. PDL is the fibrous connective tissue and contains heterogeneous cell populations including the fibroblast, osteoblast, blood vessel cells, nerve cells, and mesenchymal stem cells [21-23]. It is thought that these PDL cells have an ability to maintain the structure of the periodontium [24]. If an implant possessing a periodontal ligament could be developed, these problems would be resolved. Therefore, much attention has been given to preparing the artificial root possessing the PDL. For instance, PDL cell culture on the titanium artificial root and the periodontal tissue regeneration on the implant have been studied [25-38], though so far the complete reconstruction of PDL has not been yet achieved. Buser et al. [26] reported that collagen fibers inserted into the cementum-like tissue on the implant surface when the implant was placed in contact with adjacent tooth periodontium, and the result suggested that the retained tooth root could serve as a source for cells, participating in the healing process. However, it is still unknown how is the interface between the collagen fiber and the implant surface that was bioinert inorganic material. Nyman et al. [24] demonstrated that PDL cells have the capacity to form periodontium. Buser and Warrer [27] demonstrated that a cementum layer inserting collagen fiber was formed around the titanium implant, contacted with neighbor retained natural tooth root. Boyko et al. [32] showed that cultured PDL cells could create a new PDL when reimplanted with a demineralized root. But in their reports, the regenerated PDL like tissue could be seen only near the retained tooth root. Van Dijk et al. [33] demonstrated that cell seeding of cultured PDL cells could produce a new connective tissue attachment on a planed root surface. Choi [34] and Urabe [35] reported that the connective tissue inserted into the regenerated cementum was perpendicularly arranged with the titanium implant. However, they did not

mention about the interface between titanium and the regenerated periodontium. As titanium is an inorganic material and is likely to give low bioaffinity with an organic tissue such as a collagen fiber or a cementum, the author proposed a hybrid implant consisting of an inorganic implant covered with an organic material including cells seeded on its surface to make a regeneration of periodontium easy.

Recently, it was proposed the concept of tissue engineering [39,40] that means the technology of regeneration of tissue using artificial scaffold materials incorporated living cells. This technology has been applied many tissue such as skin [41,42], cartilage [43,44], cornea [45], tooth [46] and so on. It is thought that the tissue engineering has a possibility to solve many problems about donor shortage for tissue transplantation. The present work is one of the applied researches in combination with the polymer chemistry, tissue engineering and dentistry and can be worthwhile not only in the scientific but in social aspect in the future aging society.

The strategy for the hybridization is explained as follows. A titanium implant is coated with a polymer to enhance the biocompatibility. And then, PDL cells are cultured on the modified implant and the specimen is implanted into a jawbone. PDL regeneration is expected to take place around the artificial tooth root.

In Chapter 1, it was the objective to find out what type of polymer has a high affinity to titanium in the first stage of the project and to validate results for biomedical applications. The adhesive strength between titanium and various kinds of commercial polymers was evaluated to select a covering material having high affinity. Lap shear and T-peel adhesive strength and water resistance of adhesion in water at 37°C were evaluated. And the mechanism of the adhesion was investigated using electron spectroscopy for chemical analysis (ESCA) and the contact angle. From the results, poly(ethylene-co-vinyl



alcohol)(EVA) showed the highest strength. Many studies on EVA [47-49] have been referenced including the usage for biomaterials [50] and EVA has commonly been used for coating films because of its high gas impermeability [51]. EVA from the saponification of poly(ethylene-co-vinyl acetate )(EVAc) has been commonly used as a hot-melt type adhesive [52,53]. It is well known that EVAc has a high affinity to metals, and the adhesive strength is so high [54]. So, it is likely that EVA also has the high adhesiveness and strength.

In addition, the modification of titanium by  $H_2O_2$  could enhance the adhesive strength against EVA. It was based on not only an increase in surface adhesive area, but also an increase in the hydrophilicity of titanium by the production of  $Ti-OH$  [55-57]. It was concluded that EVA was selected to use the covering material with a titanium implant by its highly adhesiveness.

In Chapter 2, EVA was attempted to modify to enhance the biocompatibility of implant surfaces. As in EVA, the hydroxyl group is easy to convert into other functional groups such as carboxyl [58-60] and amino groups [61]. It is well known that biocompatibility can be improved by collagen immobilization onto surfaces having those groups [62-64]. Collagen immobilization onto several polymers has been carried out through polyioncomplex with carboxyl groups introduced by the graft polymerization of acrylic acid [64-65]. As EVA is the polymer of secondary alcohol, carboxyl groups are introduced by surface oxidation. The results of carboxyl group introduction through ozone oxidation and collagen immobilization were shown and the difference in amount of collagen immobilization on the poly(acrylic acid) grafted EVA surface was discussed.

Chapter 3 shows the culture of PDL cells derived from a human tooth root [66] on collagen immobilized EVA, focusing on searching the most suitable condition for the culture of

PDL cells and evaluating the essential function of cells, such as cell adhesion, extension, proliferation, and production of type I collagen and fibronectine on that condition. The cell morphology on the each surface condition was also discussed.

In Chapter 4, the differentiation of PDL cells on the modified EVA surface was evaluated. PDL contains heterogeneous cell populations and has been regarded as a source of cementoblasts and osteoblasts [67]. The aim of this chapter is to control the PDL cell differentiation and to construct the highly organized hybrid implant, and to regenerate the periodontal tissues including the cementum, PDL, and the alveolar bone. Generally, periodontal ligament fibers come into the cementum or the alveolar bone, which makes the tooth root strongly fixed. Cementum was the highly calcified tissue and metabolized by cementoblasts and cementoclasts. The cementum and bone resembled to each other and still unclearness in the difference. Cementoblasts also show bonelike cells properties such as a high alkaline phosphatase activity and osteocalcin secretion, the most well-characterized marker of calcified tissues [68,69]. To induce the differentiation of PDL cells, the surface of EVA was covered with hydroxyapatite that is the major component of a bone. The methodology of hydroxyapatite immobilization and results of PDL cells differentiation cells in terms of expression of osteoblast marker proteins were discussed.

Chapter 5 described the differentiation of gingival cells toward osteoblastic cells on the hydroxyapatite immobilized EVA. Chapter 4 showed PDL cells can acquire the calcified tissue cells on the hydroxyapatite coated EVA. When it will apply to the hybrid implant, however, large number of PDL cells were needed, which cannot be easily accessible. Gingival cell doesn't have multifunctions in contrast to the PDL cell that expresses the osteoblastic properties generally. However, it was reported that gingival cells contains functionally heterogeneous fibroblast subpopulations [70] and there are several investigations that

focused on the gingival cells properties including the osteoblastic ones [71,72]. In their reports, gingival cells expressed the mRNA of bone morphogenic proteins and showed a high alkaline phosphatase ability like PDL cells and formed mineralized tissue [73]. If gingival cells can acquire the osteoblastic properties like PDL cells, their easy availability might make it possible to regenerate PDL including cementum. The results were discussed in terms of mRNA and proteins secretion of osteoblast markers.

In Chapter 6, the author investigated the regeneration of PDL using hybrid implants through the animal test and the histological evaluation. To regenerate the human periodontal ligament (PDL) around dental implants, the novel hybrid implant was prepared as follows: First, a commercial titanium implant was oxidized by 30% hydrogen peroxide. Second, poly(ethylene-co-vinyl alcohol) was coated onto the surface of the implant and ozone treatment was carried out to introduce of carboxyl groups, followed by immobilization of collagen. In addition, the collagen sponge was also immobilized around the implant by freeze-drying method. PDL cells were seeded onto the implant and cultured at 37°C for 2 weeks, and then implanted into canine's alveolar bone.

After 3 months of implantation, the implants and their surrounding tissues were recovered. Histological analysis showed the collagen fibers connected our hybrid implants with the jawbone similar to the natural teeth, however the downgrowth of the gingival tissue was observed. Therefore, a guided bone regeneration membrane was used to prevent the downgrowth of the gingival tissue. The results were given and discussed in this chapter.

In summary, this study was carried out using the methods of biomaterials, tissue engineering and dentistry to develop the newly dental implant possessing the PDL. The selection of EVA as the covered material of a current implant made it easy to enhance a

biocompatibility and to hybridize living cells to itself. From animal tests, it was confirmed to regenerate PDL around the hybrid implant. The study of PDL regeneration around hybrid implant can open new perspectives in not only oral implantology and dentistry but tissue engineering and biomaterials.

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## Chapter 1

### Adhesion between Poly(ethylene-co-vinyl alcohol)(EVA) and Titanium

#### Introduction

Implantation of artificial tooth roots has been widely performed in dental surgery to recover the lost functions of a natural tooth. A familiar method of direct implantation, the so called “osseointegrated type”, aims at fixing the root in the submaxilla [1,2] and some problems have been reported in that the direct impact of occlusal and bruxing forces has often caused absorption of the alveolar bone and loosening of the implant by excessive stress between the artificial titanium root and the jaw bone [3,4]. In contrast, a natural tooth has a periodontal ligament (PDL) between the cementum and alveolar bone, mainly consisting of active fibroblasts and collagen fibers and plays quite an important role as a shock absorber against the mastication impact and as a receptor of forces [5]. Therefore, it is desirable that artificial roots have such functions as the PDL. Many trials have been given to the culture of PDL cells onto dental materials [6-8] and to prepare the artificial root possessing the PDL [9,10], although the complete reconstruction of the PDL has not been achieved yet.

In this study, author intended to prepare a “hybrid implant” with the PDL, which means the hybridization of the human PDL using artificial biomaterials and regeneration of the PDL around them *in vitro* and *in vivo*. The strategy for the hybridization is schematically illustrated in Fig.1. First, a titanium implant is coated with polymers and surface modification is employed to enhance the biocompatibility of the polymers followed by collagen immobilization. Next, the PDL cells are cultured on the modified implant and the specimen is implanted into jawbone. PDL regeneration is expected to take place around the

artificial tooth root. The objective of this research was to find out which polymer has a high affinity to titanium in the first stage of our project and to validate the results for biomedical applications.

Many studies on poly(ethylene-co-vinyl alcohol)(EVA) have been referenced [11] including the usage for biomaterials [12] and coating films because of its high gas impermeability [13,14]. Hydroxyl groups in EVA are easy to convert into other functional groups such as carboxyl [15] and amino groups [16]. Biocompatibility can be enhanced by collagen immobilization onto the surfaces through the oxidation [17,18]. It is expected that periodontal ligament cell culture on collagen-immobilized EVA makes it easy to regenerate periodontal ligaments.

The choice of polymer for coating onto titanium in Fig.1 is thought to be very important. EVA from the saponification of poly(ethylene-co-vinyl acetate )(EVAc) has been commonly used as a hot-melt type adhesive [19,20]. It is well known that EVAc has a high affinity to metals, and the adhesive strength is so high [21]. So, it is likely that EVA also has high adhesiveness and strength. The adhesiveness between metals and several polymers has already been reported [22,23], although few studies have focused on the mechanisms of their adhesion. This chapter will focus on the selection of a polymer with a high affinity to titanium and on the adhesiveness of EVA-titanium, and the mechanism of adhesion will be discussed in detail.

## **Materials and methods**

### **Materials**

EVA ( $M_n = 15,000$ ,  $3\phi \cdot 4\text{mm}$  pellets and  $200\ \mu\text{m}$  thick films) having 56 mol% of vinyl alcohol content was kindly supplied by Nippon Synthetic Chemical Industry Co. Ltd. (Osaka,

Japan) and High-density poly(ethylene) and poly(styrene) were from Aicello Inc. (Aichi, Japan). Poly(urethane) (Pellethane<sup>®</sup>) and poly(vinyl alcohol)(PVA) (Mw = 75,000) were from Dow Chemical Co. (Midland, MI, USA) and Unitika Ltd. (Osaka, Japan), respectively. Acid cured gelatin was kindly supplied by Nitta Gelatin Co. Ltd. (Osaka, Japan) and cellulose acetate, hydrochloric acid, and sodium hydroxide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Pure titanium sheets (JIS H4600, Grade 1, TR270-H, 0.1mm thick) from Fukuda Metal Foil & Powder Co., Ltd.( Kyoto, Japan) were used after ultrasonic cleaning in distilled water. All other materials were of chemical reagent grade and used without further purification.

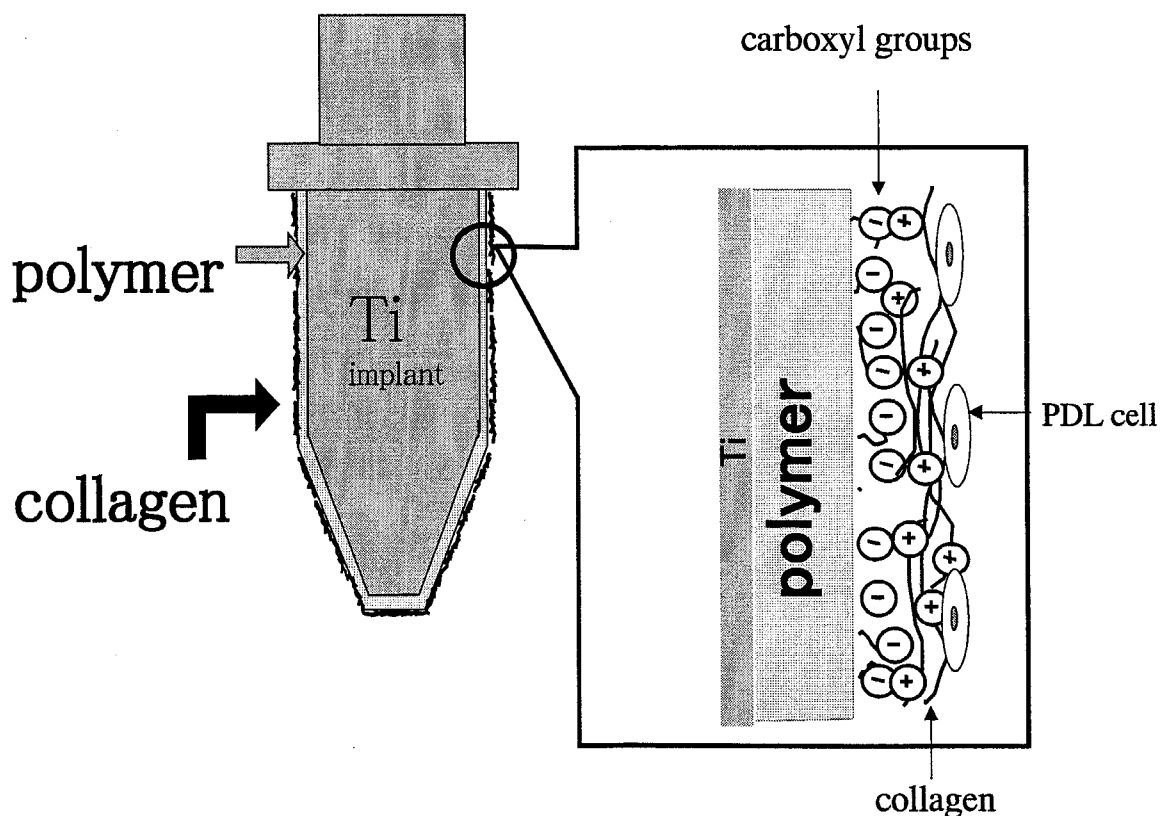


Fig.1 A schematic illustration of a hybrid dental implant

## Adhesive tests of titanium with polymers

### A. Shear adhesive strength

Lap shear adhesive tests of titanium with polymers were carried out to evaluate the physicochemical affinity among them. Five kinds of hydrophilic polymer (EVA, PVA, cellulose acetate, gelatin, and poly(urethane)) and two hydrophobic polymers (poly(ethylene) and poly(styrene)) were used. 0.05 g of thermoelastic polymer pellets such as poly(ethylene), poly(styrene), poly(urethane), and EVA were put between two titanium sheets cut into 10×50 mm and were pressed at 200°C, 20MPa pressure for 10 minutes and cooled down to 25 °C. To obtain the same adhesive area, poly(tetrafluoroethylene)(PTFE) sheet spacers of 10 (lap length)×3 (width)×0.1 (thickness) mm (Fig.2) were used. When cellulose acetate, gelatin and PVA were tested, they were dissolved in the given solvent at a concentration of 10 w/w % and each solution was put between the two titanium sheets and the solvent was evaporated at 150°C for 24 h, because these polymers showed no melting points. The solvents used were as follows : a mixture of n-propanol and water (70 / 30, weight, for EVA), water (for PVA and gelatin), and acetone (for cellulose acetate). The lap shear adhesive strength ( $\tau$ ) was evaluated by the following equation

$$\tau = P_{\max}/(Lb)$$

where  $P_{\max}$ , L, and b are the maximum tensile strength at break, the lap length, and the width of the test specimens, respectively. Five test pieces for each sample were used to evaluate the adhesive strength using a tensile tester (Autograph<sup>®</sup> DSS 2000, Shimadzu Inc., Kyoto, Japan) at 25 °C under a tensile speed of 2 mm/min and the results were assessed according to the Student's t-test.

### B. T-Peel test

The Tpeel strength (G) was calculated by the following equation



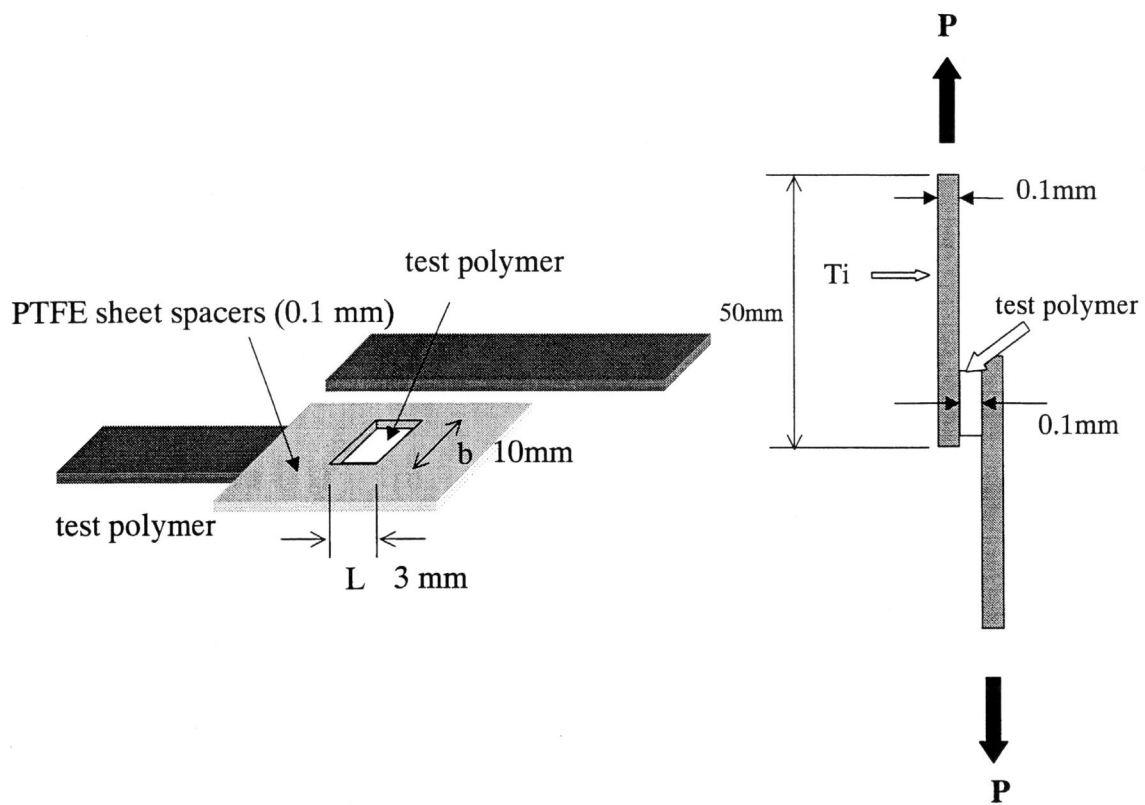


Fig.2 A schematic illustration of a specimen of the lap shear test

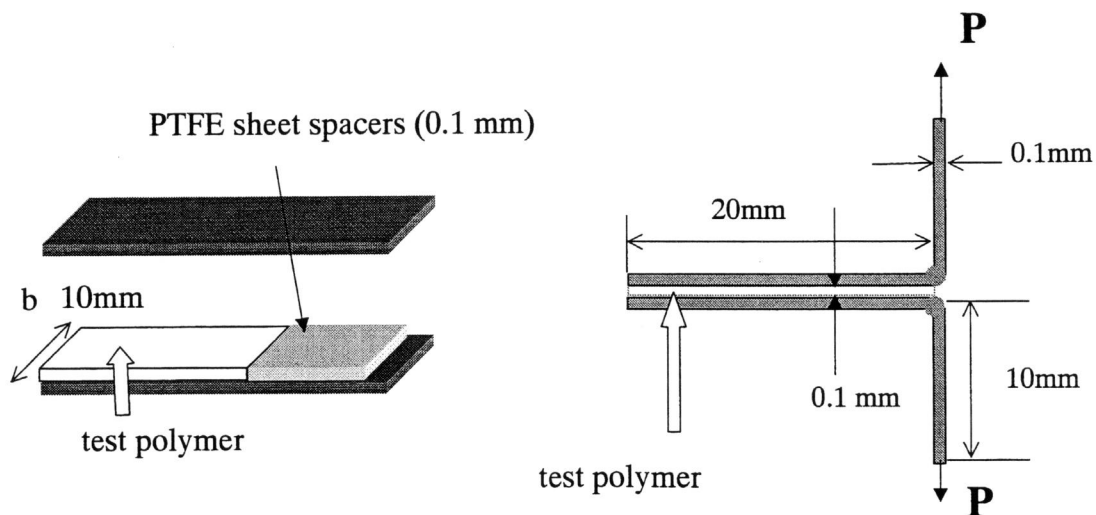


Fig.3 A schematic illustration of a specimen of the T-peel test

$$G = 2 P_{ave} / b$$

where  $P_{ave}$  and  $b$  are the average force required to peel off a specimen and the width of the specimen, respectively. Measurement was undertaken in the same manner as the shear adhesive strength test except for the adhesive area ( $20 \times 10$  mm) (Fig.3) and tensile speed (50 mm/min).

#### **Water resistance of adhesion**

The water resistance of adhesion was determined as follows: The test specimens prepared in the same manner as described above were immersed in distilled water for up to 1 month at 37 °C and the adhesive strength was evaluated at a given period of immersion.

#### **Surface analysis**

##### **Contact angle measurement**

The contact angles of titanium and polymers to water were measured with a contact angle meter (CA-X, Kyowa Interface Science Co., Ltd, Saitama, Japan) at 25 °C in order to investigate their surface hydrophilicity. The specimens were cut 20 mm in diameter from a molded film with a thickness of 200  $\mu$ m and stored in acetone to remove the fatty stains and, before measurement, rinsed with distilled water and dried. A water droplet of 0.2  $\mu$ l was placed on a specimen and the contact angle was measured after 1 minute. Five readings at different points were averaged for each specimen.

##### **Electron spectroscopy for chemical analysis**

0.05 g of EVA was put between titanium and PTFE sheet and pressed into ca. 0.1 mm in thickness at 200 °C and 20 MPa pressure. After cooling down to 25 °C, the EVA sheet was mechanically peeled off and then the EVA surfaces at both sides of the titanium and PTFE were analyzed by electron spectroscopy for chemical analysis (ESCA, ESCA-850V, Shimadzu Ltd., Kyoto, Japan). In addition, the intact EVA film surface used in the contact angle

measurement sample was analyzed for comparison. O1s / C1s intensity ratio of EVA was evaluated to compare the surface concentration of the OH group.

#### **Surface modification of titanium**

Titanium was oxidized with 30 w/w % aqueous hydrogen peroxide solution for 24 h at 60 °C, according to the method reported by Tengvall [24] and Ohtsuki [25]. Changes in the surface morphology of titanium were observed by scanning electron microscopy (SEM, S-2380 N, Hitachi Ltd., Tokyo, Japan) and their average surface roughness (Ra) was measured with a surface texture and contour measuring instrument (Surfcom 480A, Tokyo Seimitsu Co., Ltd., Tokyo, Japan). Reaction products on titanium by oxidation were analyzed by ESCA spectra of O1s core level. After adhesion to EVA, the lap shear adhesive strength and peeling strength were measured for modified titanium sheets.

### **Results and discussion**

#### **Lap shear adhesive strength**

The results of the lap shear adhesive tests of various polymers to titanium sheets are given in Table 1. It can be seen that in the case of the melt adhesion method, hydrophobic polymers such as poly(ethylene) and poly(styrene) have almost no adhesiveness to titanium, which will be discussed later. In contrast, hydrophilic polymers showed a higher adhesive strength to titanium. Among the polymers investigated, EVA gave the highest value of adhesion (9.87 MPa). Generally, “structural adhesives” were defined as those adhesives with a high affinity to materials such as wood, plastics, metals, and their composites and with adhesive strength higher than 6.9 MPa at room temperature [26]. Therefore, EVA might be one of the suitable structural adhesives, at least for titanium. The adhesive strengths of hydrophilic polymers such as gelatin and cellulose acetate, when

Table 1. Comparison of lap shear adhesive strength between titanium and various polymers.

polymer	method	Lap shear strength* ( $\tau$ ) / MPa
EVA	melt adhesion	$9.87 \pm 1.13$
Poly(ethylene)	melt adhesion	$\sim 0$
Poly(styrene)	melt adhesion	$\sim 0$
Poly(urethane)	melt adhesion	$6.62 \pm 1.58$
EVA (n-propanol/ water= 70/30)**	solution adhesion	$4.64 \pm 1.66$
PVA (water)	solution adhesion	$2.75 \pm 1.04$
Cellulose acetate (acetone)	solution adhesion	$0.24 \pm 0.10$
Gelatin (water)	solution adhesion	$0.33 \pm 0.18$

\*  $\tau = P_{\max}/(Lb)$ , where  $P_{\max}$ , L, b : tensile stress at break, lap length and width of adhesion, respectively.

\*\*Solvents used are shown in the parentheses. data = average  $\pm$  S.D. (n=5)

Table 2. Contact angles of water to Ti, EVA, and PE sheets.

Sample	Contact angle / degree
Ti	$32.3 \pm 7.2$
EVA	$57.3 \pm 3.4$
Poly(ethylene)	$77.7 \pm 1.9$

Table 3. O1s/C1s of EVA surface by ESCA analysis.

Surface	O1s/C1s
Intact film	0.200
Ti side	0.299
PTFE side	0.184

prepared by the solution method, were lower than that of EVA (4.64 MPa). The differences in adhesive strength in the different manners of preparation (melt and solution adhesion) might be ascribed to the difficulty in complete evaporation of the solvents. This means that the data of the solution method are not exact. However, the polymers having no melting point are well reflected as being a disadvantage in the affinity with titanium.

A time course of the lap adhesive strength of titanium with EVA and poly(urethane) in water at 37 °C is given in Fig.4. Clearly, the adhesive strength of poly(urethane) decreased with an increase in time. But no change was observed in EVA. In the case of poly(urethane), the infiltration of water to the interface leads to separation with the titanium. In contrast, when EVA was used, the penetration speed of water into the interface was slower than poly(urethane) because of its high affinity to titanium and fracture took place only in the EVA layer, quite different from that with poly(urethane). It is thought that the water molecules that penetrated through the interface between EVA and titanium were sorbed in the EVA layer due to its high hydrophilicity and did not localize at the interface to maintain the higher adhesive strength.

#### Surface analysis

The contact angles of titanium and polymers with water are shown in Table 2. The contact angle of poly(ethylene) is 77.7 degrees, quite different from that of titanium (32.3 degrees), which suggests a difference in their surface free energies and low adhesion strength. The contact angle of EVA was lower than that of poly(ethylene) but higher than that of titanium. The difference of contact angles between EVA and titanium cannot adequately explain the higher adhesion strength of EVA.

Therefore, ESCA analysis was carried out in order to investigate the reason and the results are shown in Table 3. As can be seen, O1s / C1s of EVA of the titanium side (0.299) is

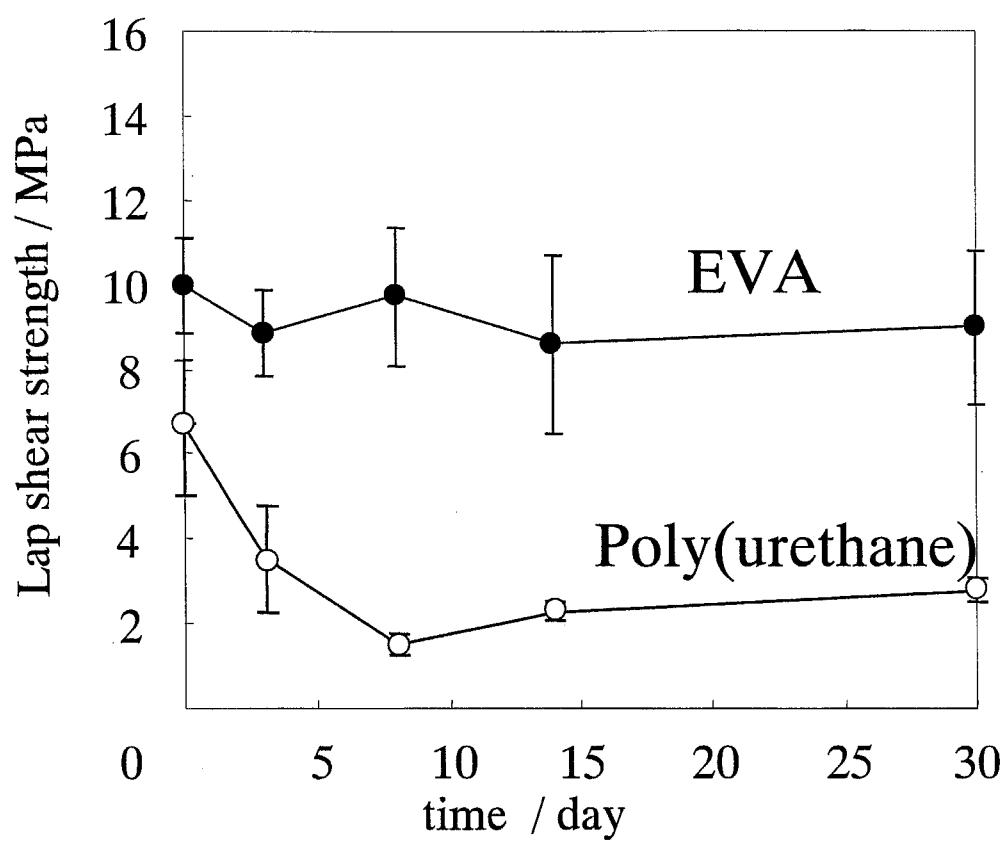


Fig.4 Change in lap shear strength of EVA and poly(urethane) with titanium sheets at 37 °C.

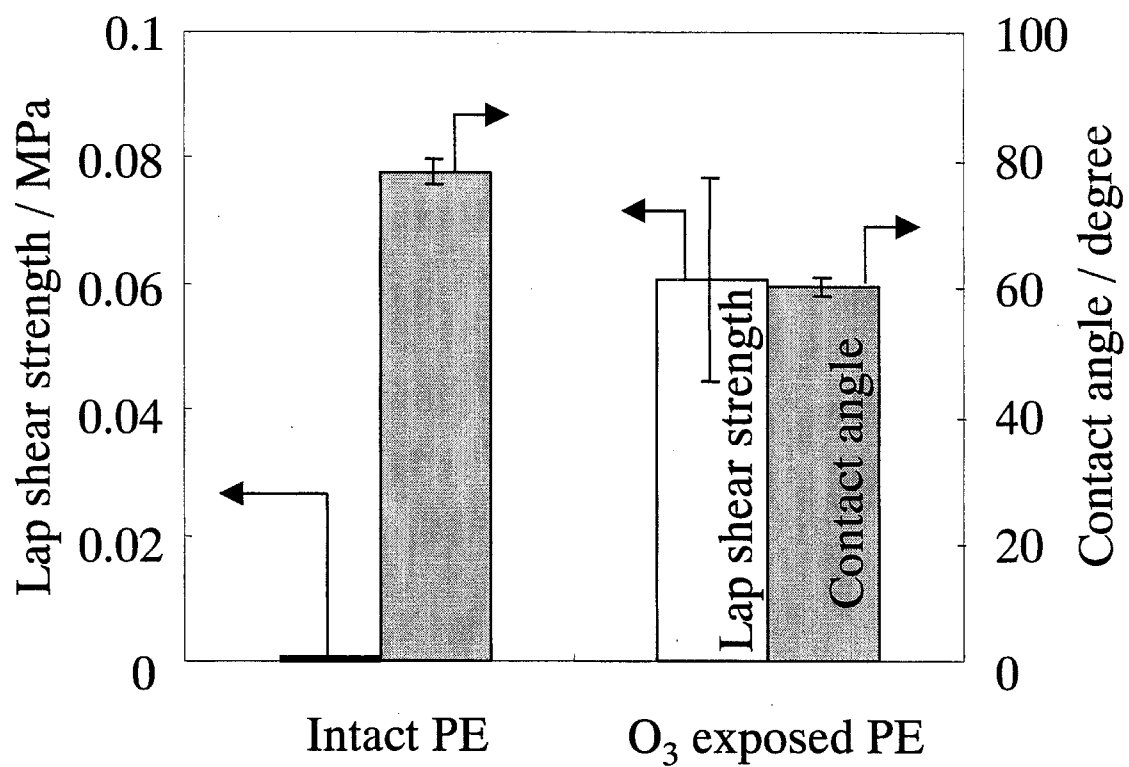


Fig.5 Effect of O<sub>3</sub> exposure to poly(ethylene)(PE) on the lap shear strength with Ti and contact angle with water.



far higher than that of the PTFE side (0.184) and the intact EVA film surface (0.200), indicating that OH groups in EVA were heterogeneously distributed in the adhesive layer during the adhesion process, according to the surface free energy of the counter materials. OH groups of EVA sheets were hidden from the surface into the inner layer as shown in Table 3, and rearranged when EVA was melted. It was suggested that during the adhesion process, the OH groups of EVA faced the titanium to make the free energy lower.

Generally, adhesives for metals are required to be hydrophilic. Poly(ethylene) films were exposed to ozone gas at a rate of 0.5 g/h for 8 h at 70 °C and carboxyl groups were introduced on the surface to enhance hydrophilicity as is described in our previous report [22]. The lap adhesive strength of titanium with modified poly(ethylene) was measured in the same way as mentioned above and the results are given in Fig.5 with those of the contact angles. The decrease in contact angle by ozone exposure was accompanied with the considerable enhancement of adhesive strength, which suggests the importance of hydrophilicity of adhesives.

#### **Surface modification of titanium**

Morphological change on the titanium surface treated with hydrogen peroxide was observed and the results are shown in Fig.6. Obviously, the surface of the titanium was significantly damaged by hydrogen peroxide and the surface roughness Ra changed from 0.02  $\mu\text{m}$  to 0.29 $\mu\text{m}$ .

The results of lap shear adhesive and peeling strength between EVA and titanium treated with hydrogen peroxide are given in Fig.7. Clearly, both strengths also increased by surface modification ( $p < 0.05$ ).

Fig.8 shows the relationship between the surface roughness and tensile shear adhesion strength when prepared by mechanical polishing (using sandpapers having

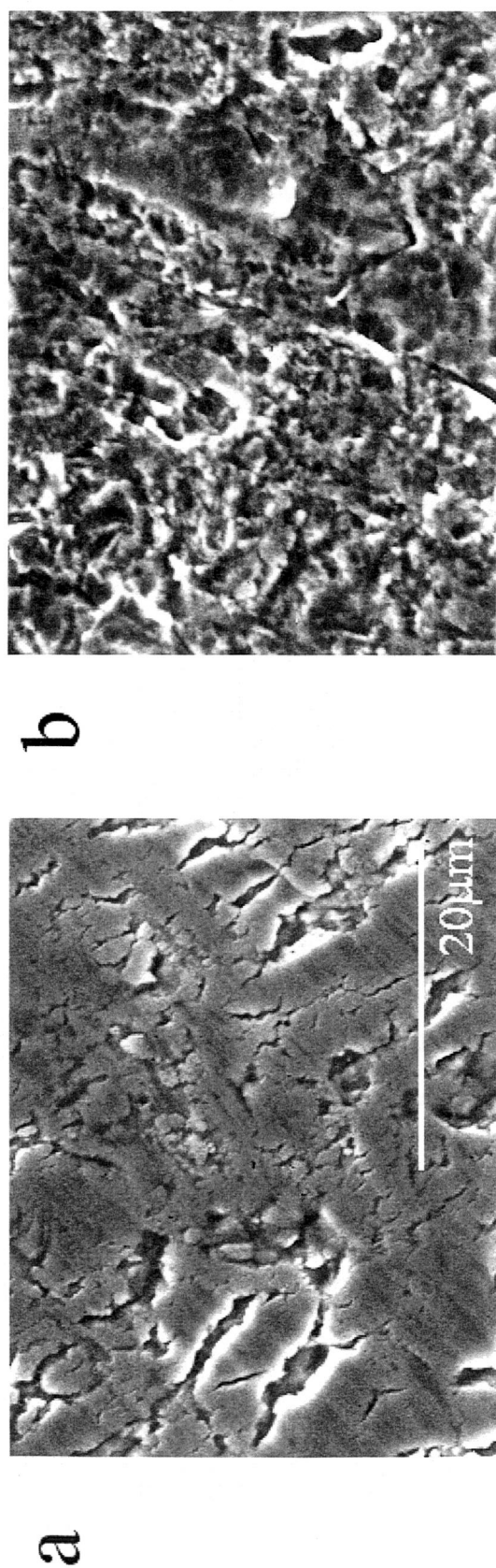


Fig. 6 SEM photographs of (a) intact and (b) H<sub>2</sub>O<sub>2</sub> treated Ti sheets

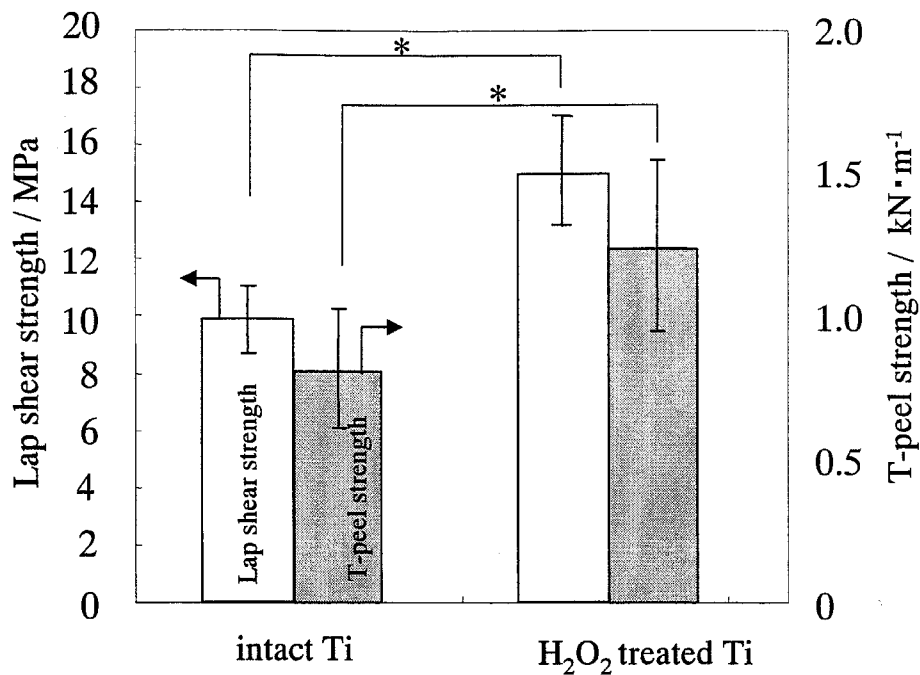


Fig.7 Effect of H<sub>2</sub>O<sub>2</sub> treatment on the lap shear and T-peel strength between Ti and EVA.

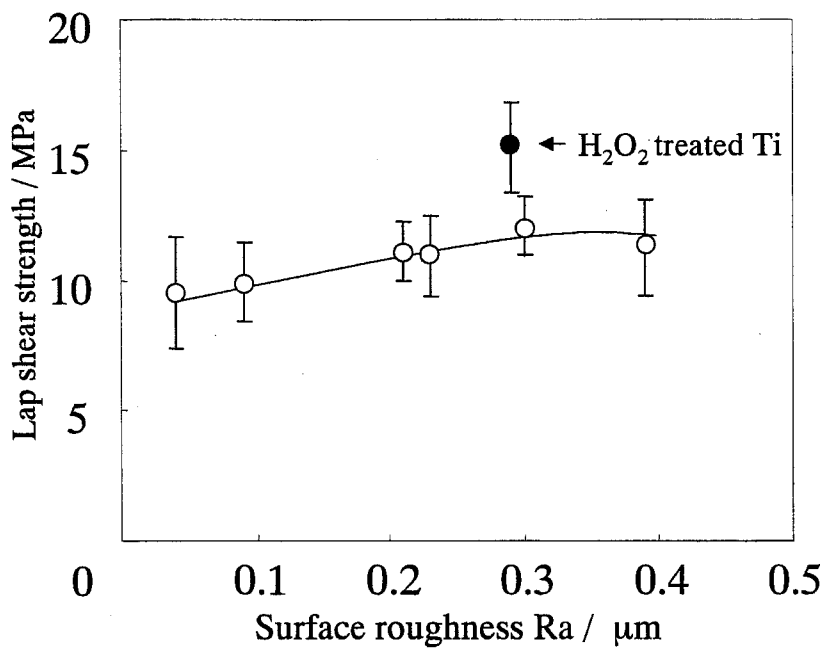


Fig.8 Effect of surface roughness of (○) : mechanically polished Ti and (●) : H<sub>2</sub>O<sub>2</sub> treated Ti on lap shear strength

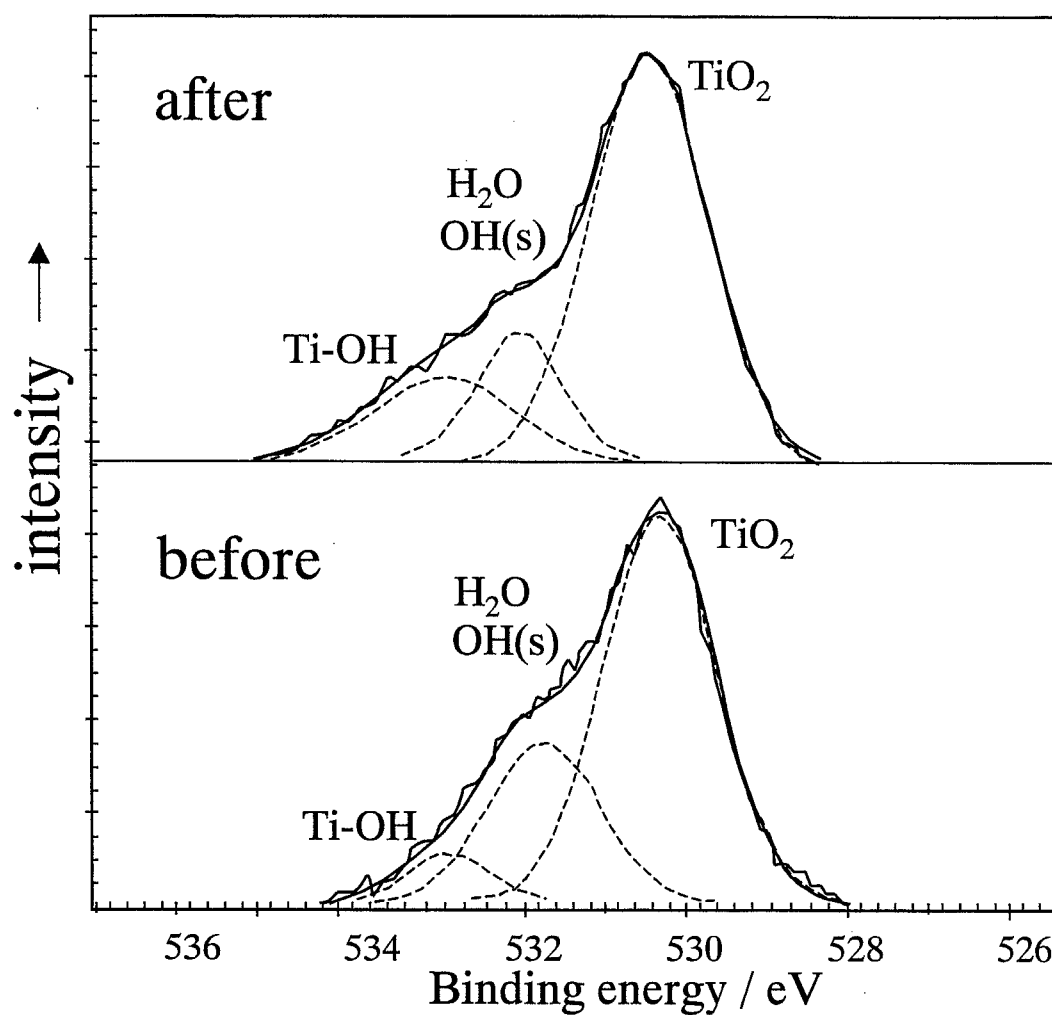


Fig.9 Core level O 1s spectra of Ti surface before and after H<sub>2</sub>O<sub>2</sub>treatment

different roughness). The surface roughness slightly affected the adhesive strength within Ra that tested the surface roughness. Therefore, the enhancement of adhesive strength in modified titanium could not be ascribed to only Ra.

ESCA spectra (O1s) of the surfaces of modified titanium are given in Fig.9. The spectra should be assigned to three different types of oxygen atoms (1) O in  $\text{TiO}_2$  (529.5), (2) O in acidic TiO-H groups and physisorbed  $\text{H}_2\text{O}$  (531.8), and (3) O in basic Ti-OH groups (533 eV) [27,28]. According to the report by Boehm [29], an acidic OH group ( $\text{pK}_a = 2.9$ ) is doubly coordinated with titanium atoms, whereas a basic OH group (Ti-OH) ( $\text{pK}_a = 12.7$ ) is singly co-ordinated. It has been shown that the peak intensity of basic Ti-OH groups increased from 7.32 to 16.3 % (by the peak area analysis) after the treatment with hydrogen peroxide, suggesting the production of new Ti-OH by oxidation. It was concluded that surface OH groups introduced by hydrogen peroxide enhanced the hydrophilicity on the surface of titanium, leading to an increase in hydrogen bondings between EVA and titanium and in adhesive strength.

## Conclusion

EVA showed the highest adhesiveness to titanium among the various polymers investigated, due to the highest electrostatic interaction with titanium. No change in the adhesive strength between EVA and titanium was observed up to 1 month, even in water at 37 °C. Oxidation of titanium with hydrogen peroxide produced new Ti-OH groups and enhanced the adhesive strength.

These results suggested that the high affinity of titanium with EVA and high biocompatibility of EVA with periodontal ligament cells make regeneration of a periodontal ligament around an artificial dental root possible.

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## **Chapter 2**

### **Surface Modification of EVA;**

#### **Introduction of Carboxyl Groups and Immobilization of Collagen**

##### **Introduction**

Implantation of the artificial tooth root has been widely performed in dental surgery to recover the lost functions of a natural tooth. The direct impact of occlusal and bruxing forces, however, often has caused absorption of the alveolar bone and loosening of the implant, because the artificial root made of titanium [1,2] is meant to be fixed directly in a jawbone. On the other hand, the root of a natural tooth is covered with a periodontal ligament (PDL), mainly consisting of active fibroblasts and collagen fibers, [3,4] and plays quite an important role as a shock absorber [5] against the mastication impact and as a receptor of forces. Therefore, much attention has been given to preparing the artificial root possessing the PDL. For instance, PDL cell culture on the titanium artificial root and periodontal tissue regeneration on the implant have been studied [6-12], though so far the complete reconstruction of PDL has not been yet achieved.

In this chapter, I attempted to modify poly(ethylene-co-vinyl alcohol) (EVA) to enhance the biocompatibility of titanium surfaces, which show high affinity with metal or ceramics and are easily coated onto implants. Many studies on EVA [13-15] have been referenced including the usage for biomaterials [16], however, few reports have focused on the surface modification of EVA for biomedical applications. EVA has commonly been used for coating films because of its high gas impermeability [17]. As in EVA, the hydroxyl group is easy to convert into other functional groups such as carboxyl [18-20] and amino groups [21].

It is well known that biocompatibility can be improved by collagen immobilization onto surfaces having those groups [22-24], therefore, it is expected that periodontal ligament cell culture on collagen-immobilized EVA makes it easy to regenerate periodontal ligaments. This chapter refers to the surface modification of EVA, carboxyl group introduction and collagen immobilization.

## **Materials and methods**

### **Materials**

Commercial EVA films having 56 mol% of vinyl alcohol content and a thickness of 200  $\mu\text{m}$  were kindly supplied by Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan). High-density polyethylene (HDPE) films with a thickness of 35  $\mu\text{m}$  were donated by Aicello Inc. (Toyohashi, Japan). These films were cut into 10 or 20 mm diameter and stored in acetone at 25  $^{\circ}\text{C}$  until usage. One w/w % type I collagen solution (pH 3.7, in 5 mM phosphoric acid) and gelatin were kindly supplied by Nitta Gelatin Co. Ltd. (Osaka, Japan), where these proteins have an isoelectric point of 9.0. Ninhydrin, hydrindantin anhydrous, 2-methoxyethanol, and acrylic acid (AAc, free acid) were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). Acrylic acid was redistilled at 52  $^{\circ}\text{C}$  in vacuum (3 mmHg) before usage and stored at -20  $^{\circ}\text{C}$ . All other reagents were of chemical reagent grade and used without further purification.

### **Carboxyl group introduction**

#### **A. Ozone exposure**

In order to convert hydroxyl groups in EVA into carboxyl groups, EVA films were exposed to ozone. Ozone gas was produced by a generator (Type O-1-2, Nippon Ozone Co.

Ltd., Japan) equipped with an O<sub>2</sub> cylinder and taken into a glass bottle in a water bath at a given temperature at a rate of 1.0 L/min. A thermometer was set in the bottle and the ozone temperature was recorded. EVA or HDPE films were hung in the bottle and treated with ozone for different periods of time. The flow ratio of ozone gas was 0.5 g/h, which was determined by conventional oxidation-reduction titration. Briefly, the amount of sodium thiosulfate required for the reduction of I<sub>2</sub> into I<sup>-</sup> was measured, where I<sub>2</sub> was produced through the oxidation of I<sup>-</sup> by ozone.

## B. Graft polymerization

Graft polymerization of acrylic acid was also carried out to introduce carboxyl groups onto EVA or HDPE films. After the peroxide introduction onto the surface of these films by ozone [25] for 2 h at 47 °C (flow rate = 0.5 g/h), the films were put into 10 ml of 10 w/w% aqueous acrylic acid solution in a test tube and sealed after degassing for 15 min in reduced pressure. The test tube was immersed in a water bath at 60 °C for 1 h to allow the polymerization reaction to proceed. The poly(acrylic acid) (PAAc) grafted film was recovered after rinsing with an excess amount of distilled water at 60 °C for 16 h to remove the homopolymer from the film surface [26].

## Determination of carboxyl group produced

Surface analysis of ozone-exposed EVA films was carried out by electron spectroscopy of chemical analysis (ESCA-850V, Shimadzu Ltd. Kyoto, Japan) to confirm carboxyl group production. The amount of carboxyl groups was evaluated by simple neutralization titration. One piece of ozone-exposed EVA film 20 mm in diameter was put into 20 ml of  $1 \times 10^{-3}$  or  $2 \times 10^{-4}$  N-NaOH and the NaOH remaining was titrated with  $1 \times 10^{-2}$  or  $2 \times 10^{-3}$  N-HCl in the

presence of phenolphthalein. In addition, the amount of carboxyl groups on ozone-exposed HDPE, PAAc grafted EVA and HDPE films was determined in the same manner. In the case of grafted films, titration was performed after rinsing with distilled water at 60 °C for 16 h to remove PAAc homopolymer.

### **Collagen immobilization**

EVA and HDPE films 10 mm in diameter and having carboxyl groups were immersed in 0.5 w/w% type I collagen solution (pH 3.7, in 5mM phosphoric acid) and gently stirred at 25 °C for 16 h. After the formation of a polyioncomplex, unimmobilized collagen was removed by rinsing the films with an excess amount of phosphoric acid (pH 3.7). The amount of collagen immobilized was evaluated by the ninhydrin method [27] as follows : A piece of film was put into 2 ml of 2.5 N-HCl in a test tube, followed by hydrolyzation at 120 °C for 1 h. One ml of 5 N-NaOH, 1 ml of acetic acid buffer (containing 2M-acetic acid and 2M-sodium acetate, pH 5.5), and 1 ml of ninhydrin solution (0.8 g of ninhydrin and 0.12 g of hydrindantin anhydrous in 30 ml of 2-methoxyethanol) were added to the solution. After colorization at 120 °C for 1 min, the absorbance at 570 nm was measured at 25 °C (spectrophotometer 200-20, Hitachi Ltd. Tokyo, Japan). In this study, gelatin purified by reprecipitation in water/acetone was used for calibration.

### **Microscopic observation**

The surface morphology of films having carboxyl groups was observed in water using atomic force microscopy (AFM, Olympus NV2000, Olympus Optical Co. Ltd. Tokyo, Japan). A trigonal pyramid probe (silicon nitride, simple constant 0.02 N/m) was used under the condition of 5 nN force and contact mode in water at 25 °C.

## Results and discussion

### Determination of carboxyl groups produced

A comparison of the amount of carboxyl groups introduced onto the EVA and HDPE film by ozone exposure at 64 °C is given in Fig.1. Clearly, carboxyl groups introduced in EVA were much higher than those in HDPE and this might be due to hydroxyl groups on the EVA film. On the other hand, HDPE has no functional groups easily oxidized like hydroxyl groups.

The ESCA spectra of intact, ozone exposed, and PAAc grafted EVA film are given in Fig.2. Intact film shows two peaks at 286.0 eV and 287.5 eV, assigned to C1s in the C-H and C-O bonds, respectively. Ozone-exposed and PAAc grafted films show a new peak at 290.6 eV suggesting introduction of carboxyl groups.

The amount of carboxyl groups determined immediately after ozone exposure is given in Fig.3. The amount onto EVA films linearly increased with an increase in exposure time. Remarkable dependence on temperature suggests an exothermic oxidation reaction with ozone. Fig.4 shows results measured after rinsing by distilled water at 60 °C for 16 h to treat in the same manner as grafted films. After rinsing, far fewer carboxyl groups were detected in the films, regardless of temperature. It was suggested that degradation of EVA chains occurred on the surfaces of films leading to production of water-soluble fragments. This was confirmed by the following. Ozone-exposed EVA film (at 64 °C for 6 h) before rinsing and PAAc grafted film after rinsing were immersed in 20 ml of distilled water, pH was recorded and the results are given in Table 1. Considerable pH decrease was observed in the ozone-exposed film only. PAAc was covalently grafted onto the EVA surface and couldn't diffuse in water. On the other hand, water-soluble fragments were thought to

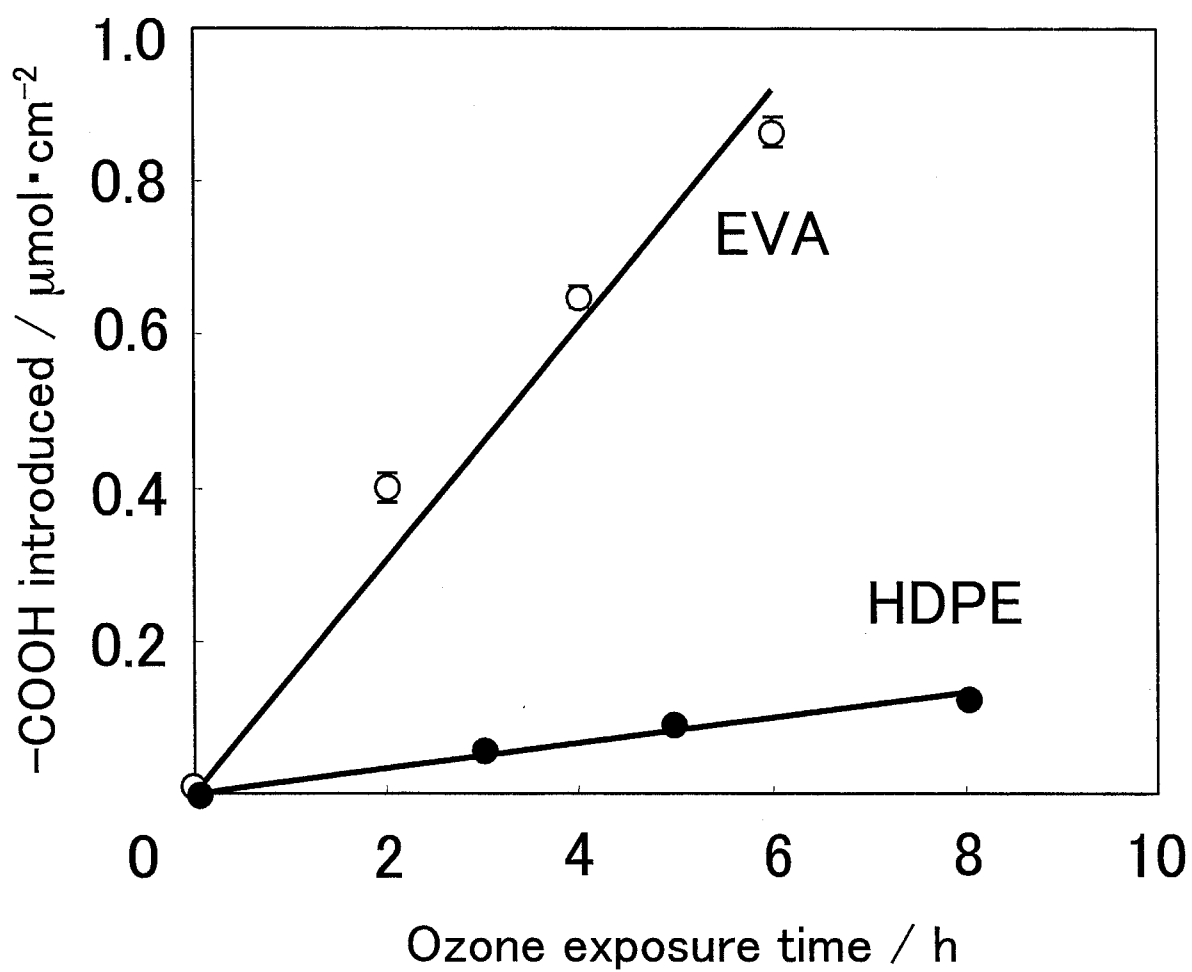


Fig.1 Carboxyl groups introduced by ozone exposure at 64°C. Error bar shows the S.D.(n=3).

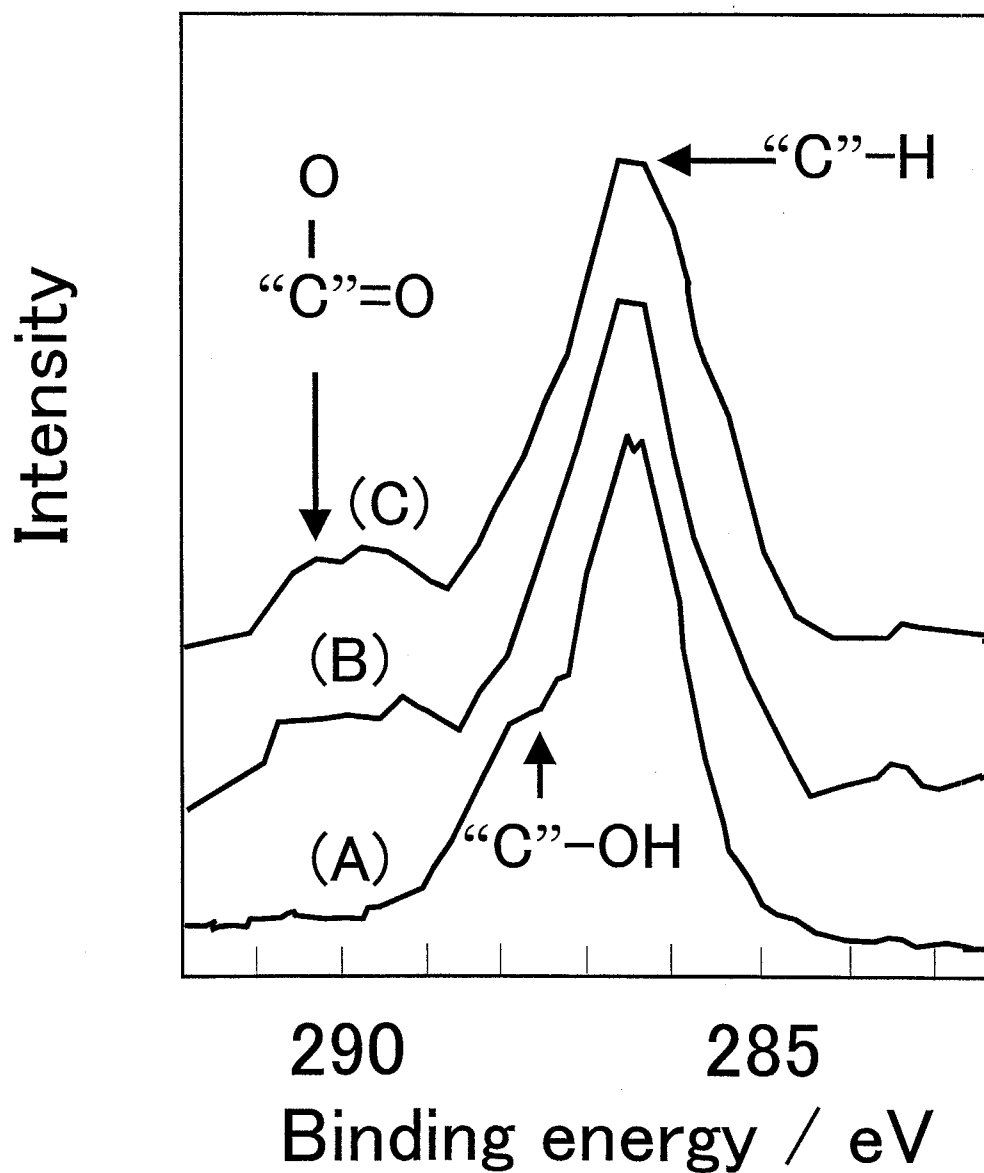


Fig.2 ESCA spectra in C1s of EVA films prepared in different manners. (A); intact, (B); ozone-exposed, and (C); PAAc grafted EVA.



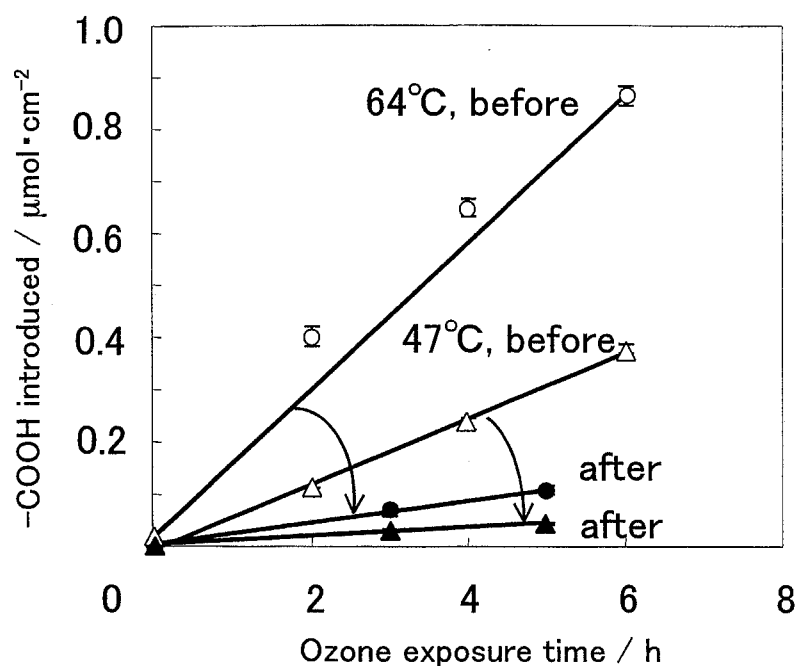


Fig.3 Temperature dependence of -COOH introduced onto EVA films by ozone exposure.

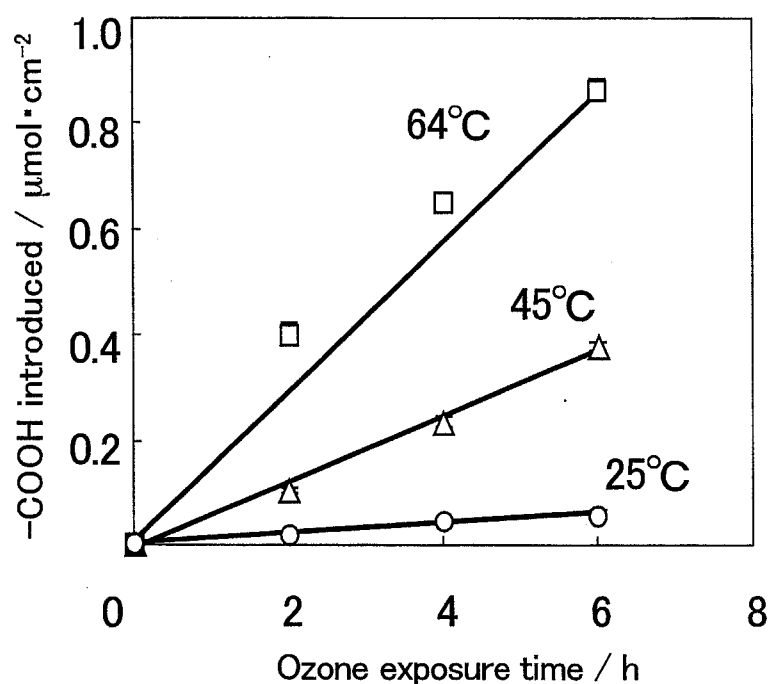


Fig.4 Decrease in -COOH introduced by rinsing with water. Ozone exposure temperature=(○); 64 °C and (△); 45 °C. Error bar shows S.D. (n=3).

decrease pH in the ozone-exposed film.

### **Collagen immobilization**

Differences in the amount of collagen immobilized onto the ozone-exposed EVA films before and after rinsing were plotted against the amount of carboxyl group in Fig.5. It was seen that the amount of collagen increased almost linearly with an increase in the amount of carboxyl groups in both systems. As described above, the amount of carboxyl groups decreased with rinsing, however, the amount of immobilized collagen also decreased after rinsing. This suggested that water-soluble fragments on EVA affected collagen immobilization. To investigate this effect the following experiment was carried out. The ozone-exposed EVA films after rinsing were dipped into 10 w/w% of PAAc solution and dried. The amount of collagen immobilized on these films was determined and is shown in Fig.6. Though PAAc was only coated and dissolved in water, the amount of collagen was markedly enhanced by the PAAc coating ( $p < 0.01$ ). It was likely that the PAAc molecules crosslinked collagen through an electrostatic interaction. Water-soluble fragments of EVA played the same role as PAAc in Fig.5.

### **Differences in collagen immobilized on ozone-exposed and grafted films**

The amounts of collagen immobilized onto ozone-exposed and grafted films are given in Table 2. In this experiment, EVA and HDPE films having almost the same amounts of carboxyl groups were prepared. Films were rinsed at 60 °C for 16 h with water before collagen immobilization. The amounts of collagen immobilized onto grafted films were much larger than those onto ozone-exposed films regardless of substrates. This might be ascribed to the difference not in surface morphology but carboxyl groups density.

**Table 1. pH of water in the presence of -COOH introduced EVA films prepared in different manners.**

Film immersed	pH of water
none	$5.73 \pm 0.06$
Grafted EVA film	$5.72 \pm 0.23$
Ozone-exposed EVA film	$4.30 \pm 0.40$

data= average  $\pm$  S.D. (n=3)

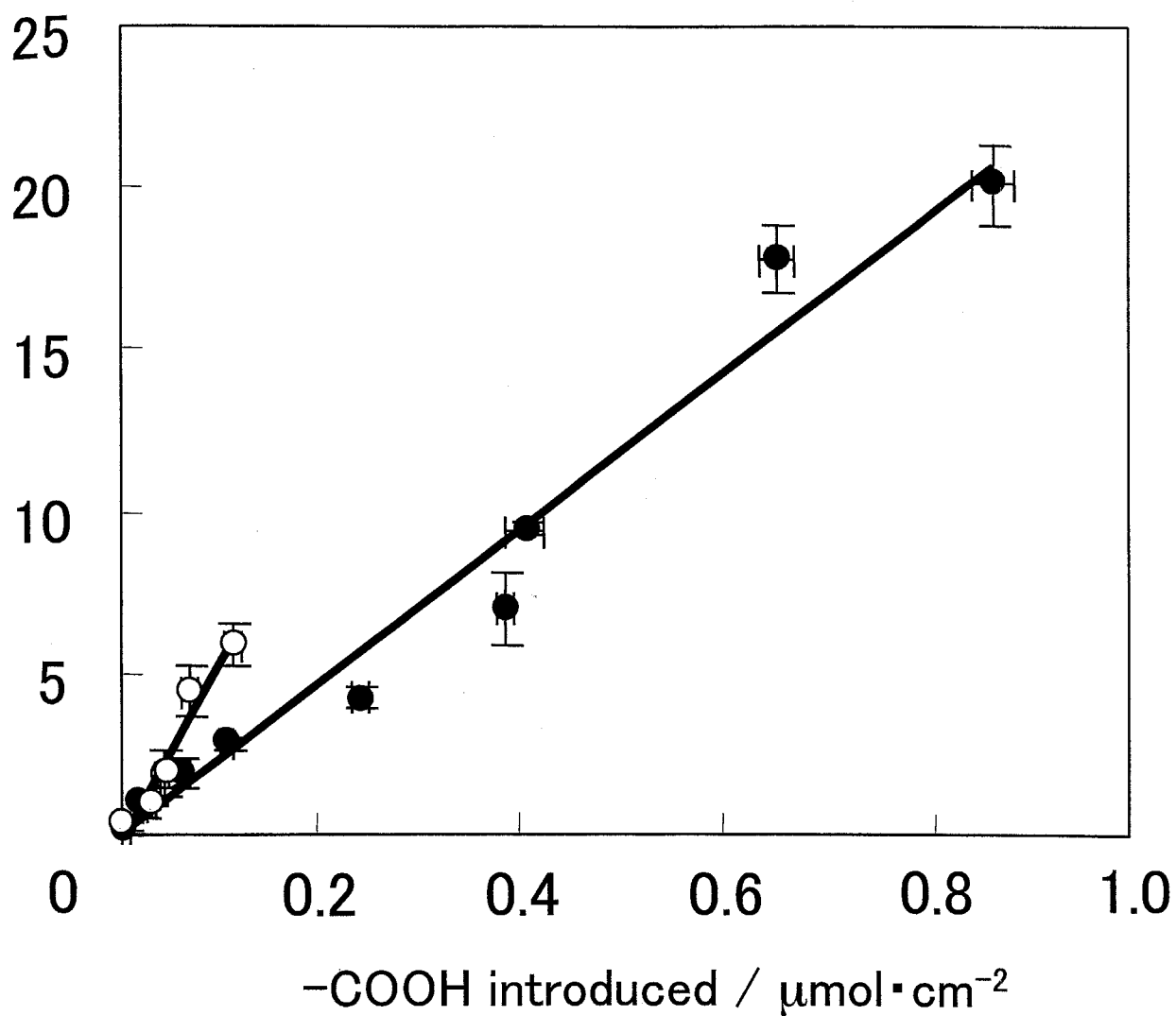


Fig.5 Relationship between -COOH introduced and collagen immobilized. (○); with and (●); without rinsing of films before collagen immobilization.

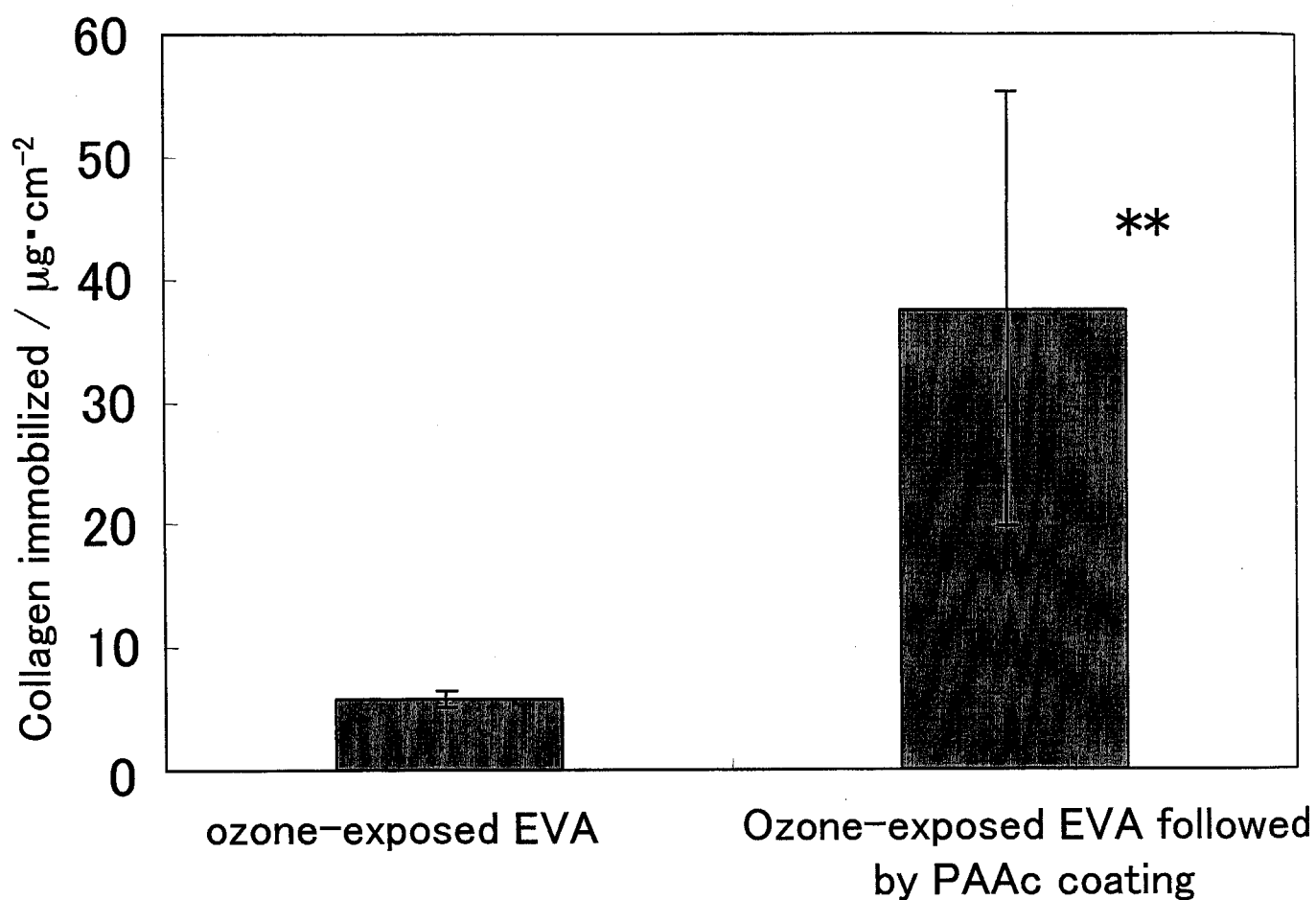


Fig.6 Effect of water-soluble fragments on the amount of collagen immobilized. Error bar shows S.D. (n=3). \*\*:  $p < 0.01$

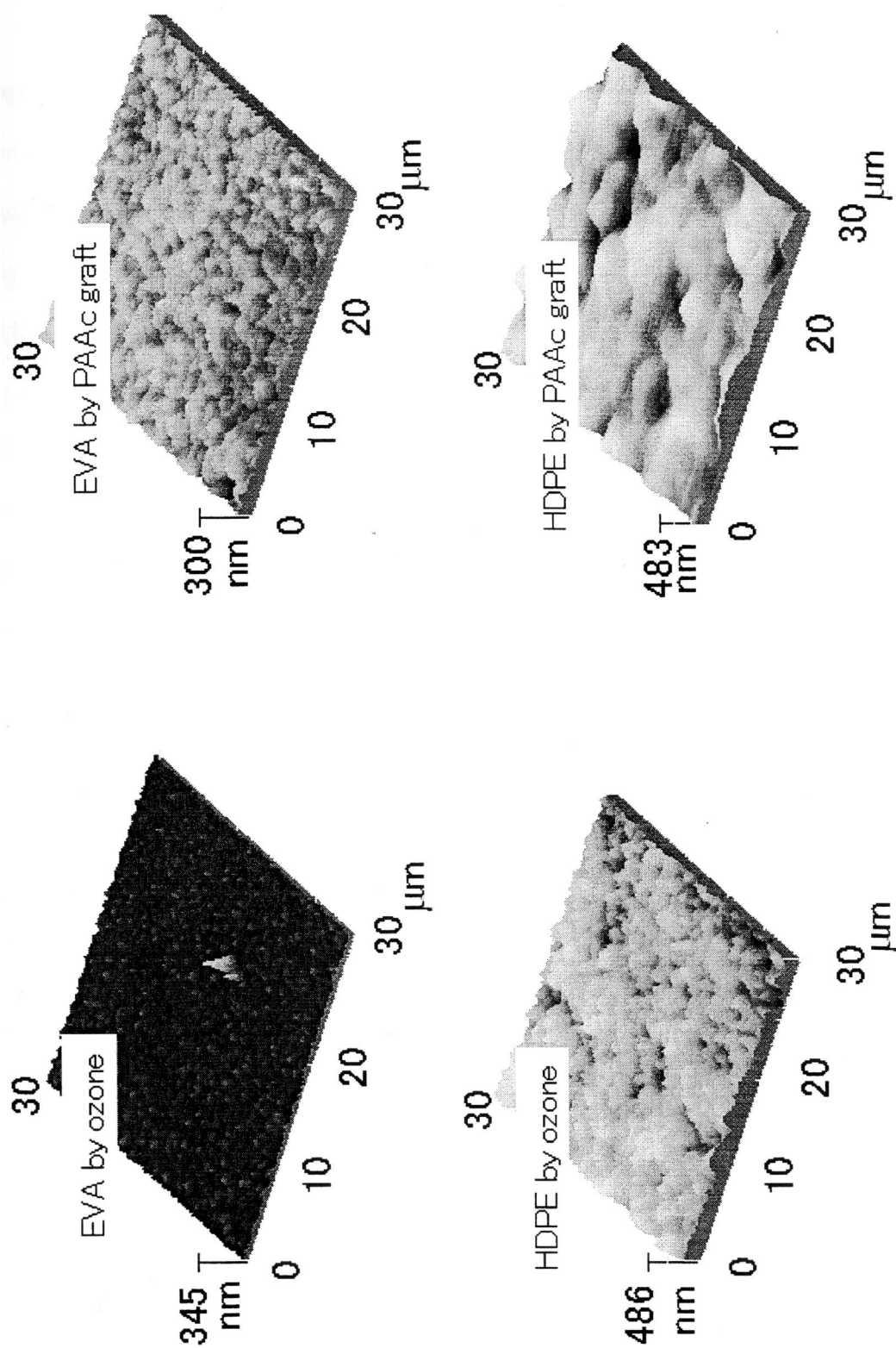


Fig.7 AFM images of EVA and HDPE surfaces in water. Carboxyl groups were introduced by ozone exposure and PAAc graft polymerization.

The surface morphology of these films was observed in water by AFM and their images are given in Fig.7. The grafted EVA surface was rougher than that of ozone-exposed EVA. The opposite result was obtained for HDPE. Therefore, it is unlikely that differences in the surface morphology between ozone-exposed and PAAc grafted substrates directly affected immobilization of collagen. Only no (head to head) or one (head to tail) carbons are in the  $\alpha$ -carbon of PAAc chains leading to an extremely high density of carboxyl groups, whereas carboxyl groups introduced by ozone were uniformly scattered all over the film surface. It is likely that differences in the charge density might influence the Coulomb force and negative charges localized on the PAAc grafted surface interacted more strongly with cationic substrates such as collagen than that scattered on ozone-exposed EVA surface.

**Table 2. Comparison of the amount of carboxyl groups and collagen immobilized onto EVA films prepared in different manners.**

	-COOH introduced ( $\mu\text{mol}/\text{cm}^2$ )	collagen immobilized ( $\mu\text{g}/\text{cm}^2$ )	
Ozone-exposed EVA	$0.116 \pm 0.028$	$8.9 \pm 1.18$	***
PAAc grafted EVA	$0.125 \pm 0.048$	$25.8 \pm 2.83$	
Ozone-exposed HDPE	$0.092 \pm 0.021$	$2.2 \pm 0.76$	**
PAAc grafted HDPE	$0.112 \pm 0.018$	$14.4 \pm 6.98$	

data= average  $\pm$  S.D. (n=3)

\*\*: $p < 0.01$ , \*\*\*: $p < 0.001$

## Conclusion

Carboxyl groups were introduced into both EVA and HDPE surfaces by ozone-exposure and PAAc graft polymerization. With ozone-exposure the amount of carboxyl groups introduced onto EVA was higher than that on HDPE. The amount of collagen immobilized onto EVA films varied with the ozone-exposure condition. Water-soluble fragments were produced by ozone-exposure to EVA and acted as a crosslinker of collagen. Differences in charge distribution of carboxyl groups affected the amount of collagen immobilization. On the basis of the above results, author will carry out the culture of periodontal ligament fibroblasts onto the collagen immobilized EVA film surface, and aim for the development of an artificial dental root with a periodontal ligament.



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## **Chapter 3**

### **Morphologic Study and Syntheses of Type I collagen and Fibronectin of Human PDL cells cultured on EVA with Collagen Immobilization**

#### **Introduction**

Collagen has been often used in the field of biomaterials associated with its natural properties such as low immunoresponse, low toxicity, and the ability to promote cellular growth and attachment [1-3]. A variety forms of collagen has been reported to some diseases involving wound dressing [4], peripheral nerve growth support [5], and guided tissue regeneration for periodontal diseases [6].

Author has attempted to regenerate the periodontal ligament (PDL) around dental titanium implants through PDL cell culture on collagen-immobilized implant (titanium + poly(ethylene-co-vinyl alcohol) (EVA) + collagen). EVA has high affinity with titanium and is so bioinert as to be applied to blood-materials such as filter and permeability membrane [7]. In chapter 2, surface modification of EVA was studied and it was found that type I collagen was easily immobilized on EVA by ozone exposure [8]. In this chapter, the purpose has been focused on searching the most suitable condition for the culture of PDL cells and evaluating the essential function of cells, such as cell adhesion, extension, proliferation, and production of type I collagen and fibronectin on that condition.

#### **Materials and methods**

##### **Materials**

A thickness of 200  $\mu\text{m}$  of commercial EVA films having 56 mol% vinyl alcohol content were kindly supplied by Nippon Synthetic Chemical Ind. Corp. (Osaka, Japan).

Type I atelocollagen (9.4 mg/ml in 5mM phosphoric acid, pH 3.7, from bovine muscle tendon) was kindly supplied by Nitta Gelatin Corp. Ltd. (Osaka, Japan). Phosphate buffer saline (PBS(-)) and Dulbecco's modified Eagle's minimum essential medium (DMEM) were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Antimycotic solution containing 10,000 unit/ml of penicillin G, 10,000 µg/ml of streptomycin, and 25 µg/ml of amphotericin B was from GIBCO BRL (N.Y., U.S.A.). L-glutamine, dextrose, ethylenediaminetetraacetic acid (EDTA), Tris-HCl buffer solution (0.05M, pH 7.6), fetal calf serum (FCS), normal goat serum, trypsin, glutaraldehyde (25 w/w% in water), and n-propanol were purchased from Nacalai Tesque (Osaka, Japan). Anti-human type I collagen and anti-human FN were from Southern Biotechnology Associates, Inc. (Birmingham, AL., U.S.A.). Anti-goat immunoglobulin rabbit antibodies and peroxidase (Peroxidase set) were obtained from Dako Corp. (Carpinteria, C.A., U.S.A.). Diaminobenzidinetetrahydrochloride (DAB) was from Merck KgaA, (Darmstadt, Germany). Tissue culture dish of 35 mm in diameter and 12 well culture plate (22 mm in diameter of each well) were from Asahi Techno Glass Corp. (Tokyo, Japan).

#### **Preparation of EVA dish**

Tissue culture dish of 35 mm in diameter was coated with 10 w/w% EVA solution in the mixture of water and n-propanol (water/n-propanol = 30/70, weight) and dried in air. After the ozone exposure, collagen was immobilized, according to chapter 2 [8]. Hereafter, EVA dishes with and without immobilization of collagen were abbreviated to "EVA+C dish" and "EVA-C dish", respectively.

#### **Preparation of EVA film**

EVA films were cut into 20 mm in diameter and store in acetone at 25 °C until usage.

Table 1. Immunostaining procedure of type I collagen and FN

- 
1. Wash the cells three times with PBS(-)
  2. Air-dry and fixation with ethanol
  3. Wash with distilled water
  4. Place in 0.3 v/v% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min at 25°C
  5. Wash with distilled water and place in tris buffer
  6. Add 3v/v% normal goat serum to the plate for 10 min at 25°C
  7. Add the primary antibodies (anti-human type I collagen or anti-FN from goat) and store at 4°C overnight
  8. Wash three times with PBS(-) (5min × 3)
  9. Add the secondary antibody (anti-goat immunoglobulin labeled with biotin from rabbit) to the plate and allow to react for 20 min at 25°C
  10. Wash three times with PBS(-) (5min × 3)
  11. Wash with tris buffer
  12. Add peroxidase labeled with streptavidin to the plate and allow to react for 20 min at 25°C
  13. Wash with tris buffer
  14. Place in DAB solution for 15 min at 25°C
  15. Stain cell nuclei with hematoxylin
  16. Mount with glycerol gelatin
-

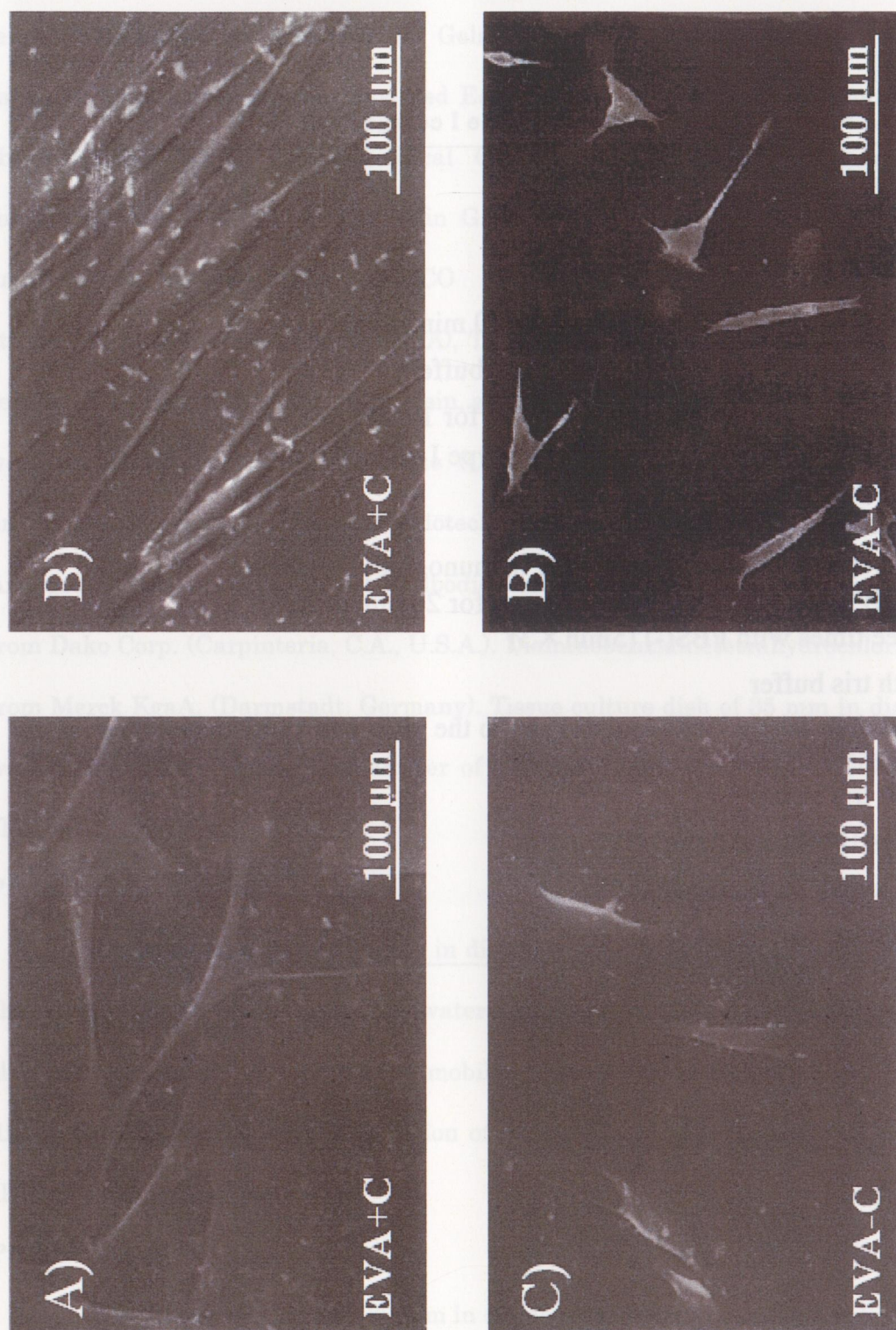


Fig.1 SEM photographs of PDL cells on EVA films for 3 and 5 days.



Collagen immobilization onto the EVA surface was carried out according to chapter 2. Hereafter, EVA films with and without immobilization of collagen were abbreviated to “EVA+C film” and “EVA-C film”, respectively.

#### **Primary culture of human periodontal ligament (PDL) cells**

Two teeth from one 19 years old healthy man were stored in PBS(-) containing 10 v/v% of antibiotic solution at 4°C immediately after the extraction. The double root apex and tooth crown tissue of periodontium piece was removed out and transferred within 6 h into a tissue culture dish with DMEM (supplemented with 0.3 mg/ml of L-glutamine, 4.5 mg/ml of dextrose, 10 v/v% of FCS, and 1.5 v/v% of antimycotic solution), followed by putting a cover glass on the tissue. The beginning of the culture, 2~3 droplets of DMEM were dropped into the side of the cover glass. Next day, 2 ml of DMEM was added carefully, and medium was exchanged 2~3 times every week. After 4 weeks, migration of PDL cells on culture dish was observed. In subculture, PDL cells were suspended in the mixture of 0.1 w/v% of trypsin and 0.025 w/v% of EDTA in PBS(-). The 4~6th passage of PDL cells were utilized for this study. Cell culture was carried out entirely at 37°C and 5 v/v% CO<sub>2</sub> under moisture, unless otherwise mentioned.

#### **PDL cell growth on EVA**

To evaluate PDL cells were seeded into EVA+C or EVA-C dishes at a concentration of  $6.6 \times 10^3 \text{ cells/cm}^2$  with 2 ml of DMEM and cultured. Growth curve for up to 5 days was recorded by counting the living cells with a hemacytometer. In this experiment culture without FCS was also carried out to evaluate clearly the effect of collagen on EVA.

#### **Evaluation of cell adhesion and extension**

PDL cells were seeded into 12 well culture plate at a concentration of  $6.6 \times 10^3 \text{ cells/cm}^2$  with 1 ml of DMEM, where EVA-C or EVA+C films of 20 mm in diameter were



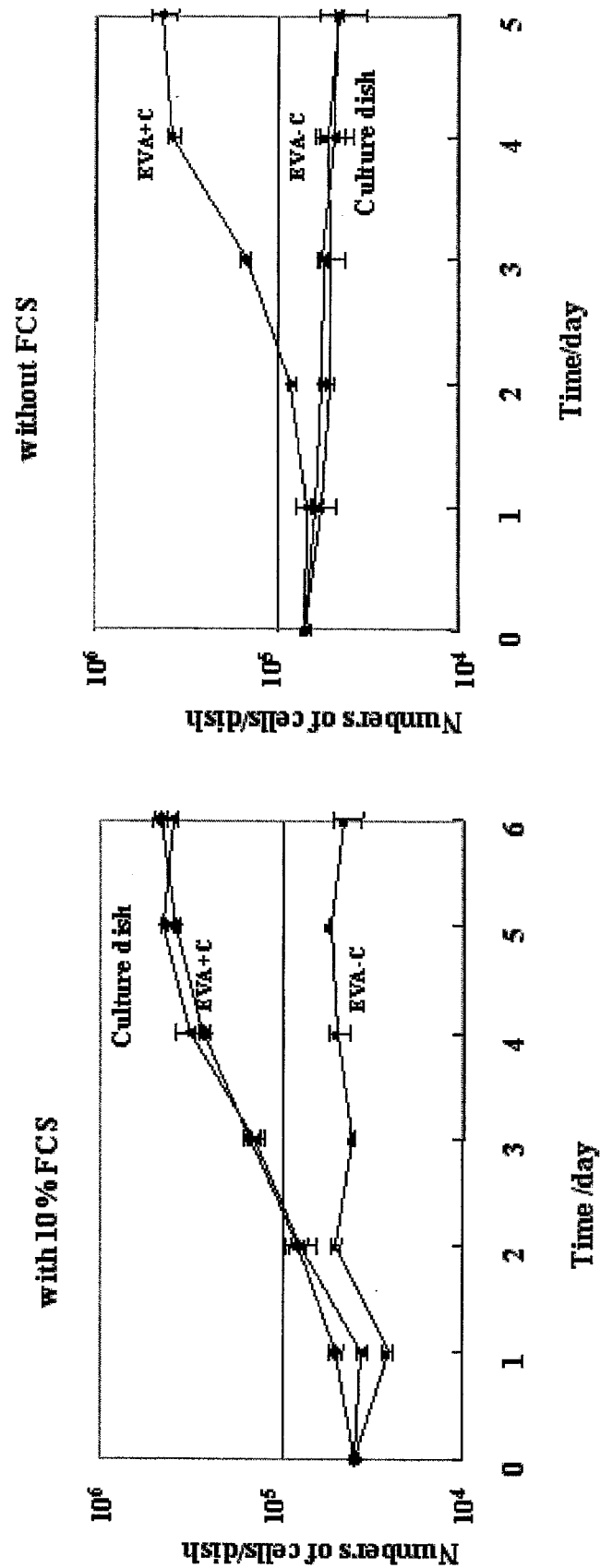


Fig.2 PDL cell growth on EVA dishes in the presence and absence of 10 v/v% FCS.

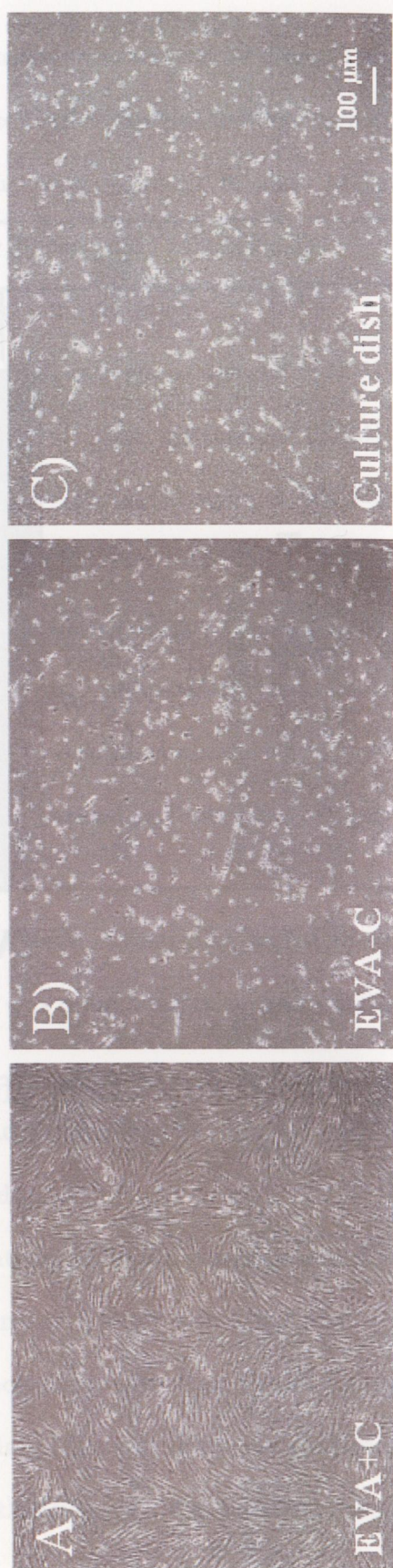


Fig. 3 Optical microphotographs of PDL cells cultured on EVA dishes without FCS for 5 days.

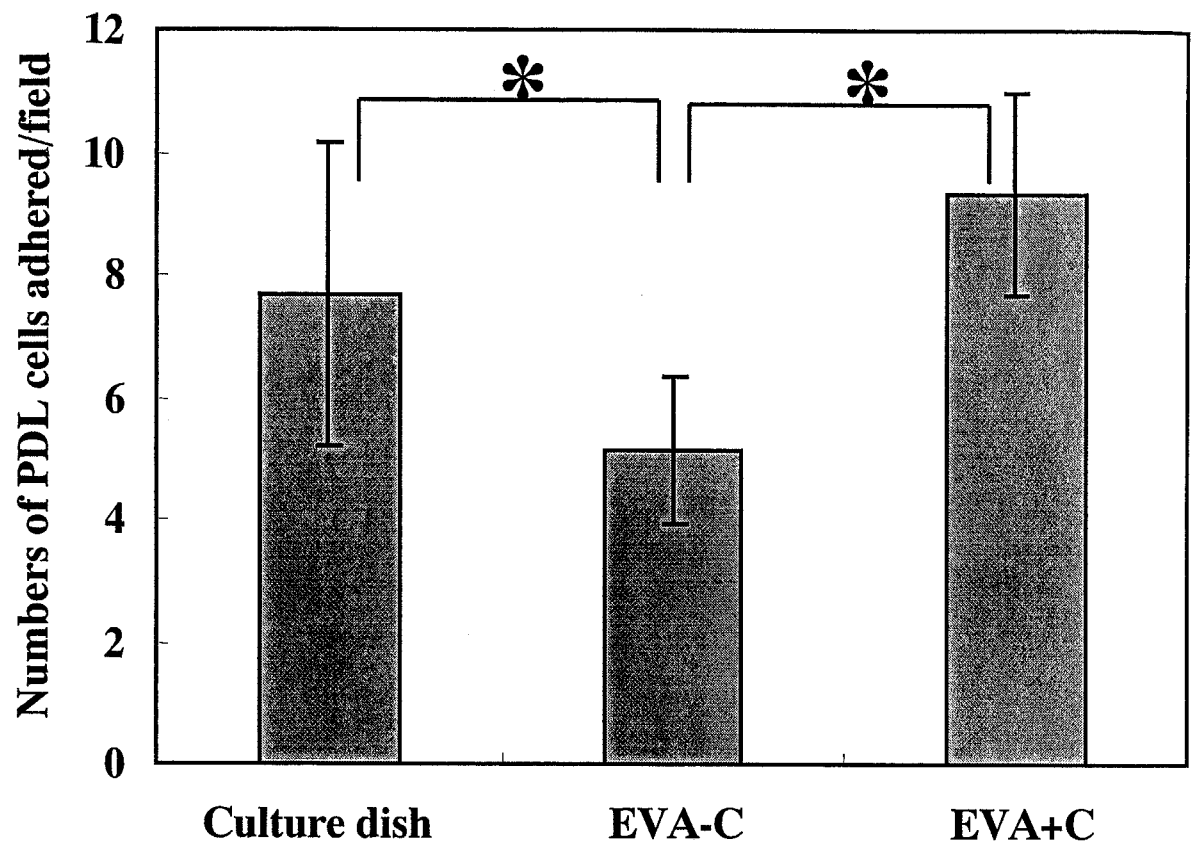


Fig. 4 Numbers of PDL cell adhered on EVA films after 1 day in the presence of 10 v/v% FCS.

placed in advance and cultured for 1 day. The numbers of adhered PDL cells were counted by hemacytometer after suspended in trypsin solution. Cells in 12 random fields (one field=0.09mm<sup>2</sup>) were counted in each samples and pseudopodia length of cells was also measured for 20 randomly selected cells.

### **Scanning electron microscopy**

The PDL cells were cultured in the same manner as described above for three or five days. Scanning electron microscope (SEM) was taken after one day fixation in freshly prepared 2.5 w/v% glutaraldehyde in PBS (-) at pH7.2 and 4 °C, rinsing, and drying.

### **Immunostaining**

The syntheses of type I collagen and FN by PDL cells were evaluated by the labeled streptavidin biotin Immunostaining method (LSAB) [9]. The PDL cells were cultured in the same manner as described above for 12 days. The cells were treated by the immunostaining procedure summarized in Table 1.

### **Statistical analysis**

Data were statistically evaluated and discussed using paired Student's t test. A P value < 0.01 was considered to be statistically significant.

## **Results and discussion**

In dental implant surgery, the healing around dental implants is aiming towards osseointegration, while the osseointegrated dental implants have not the same physiological mobility as natural tooth with a periodontal ligament (PDL), intervening between the tooth and the alveolar bone interface. The PDL serves the function of perception, buffer of the biting force, and also play a role in the metabolism of cement, bone and collagen.

Author have made some biomechanical studies on shock absorbing function of dental

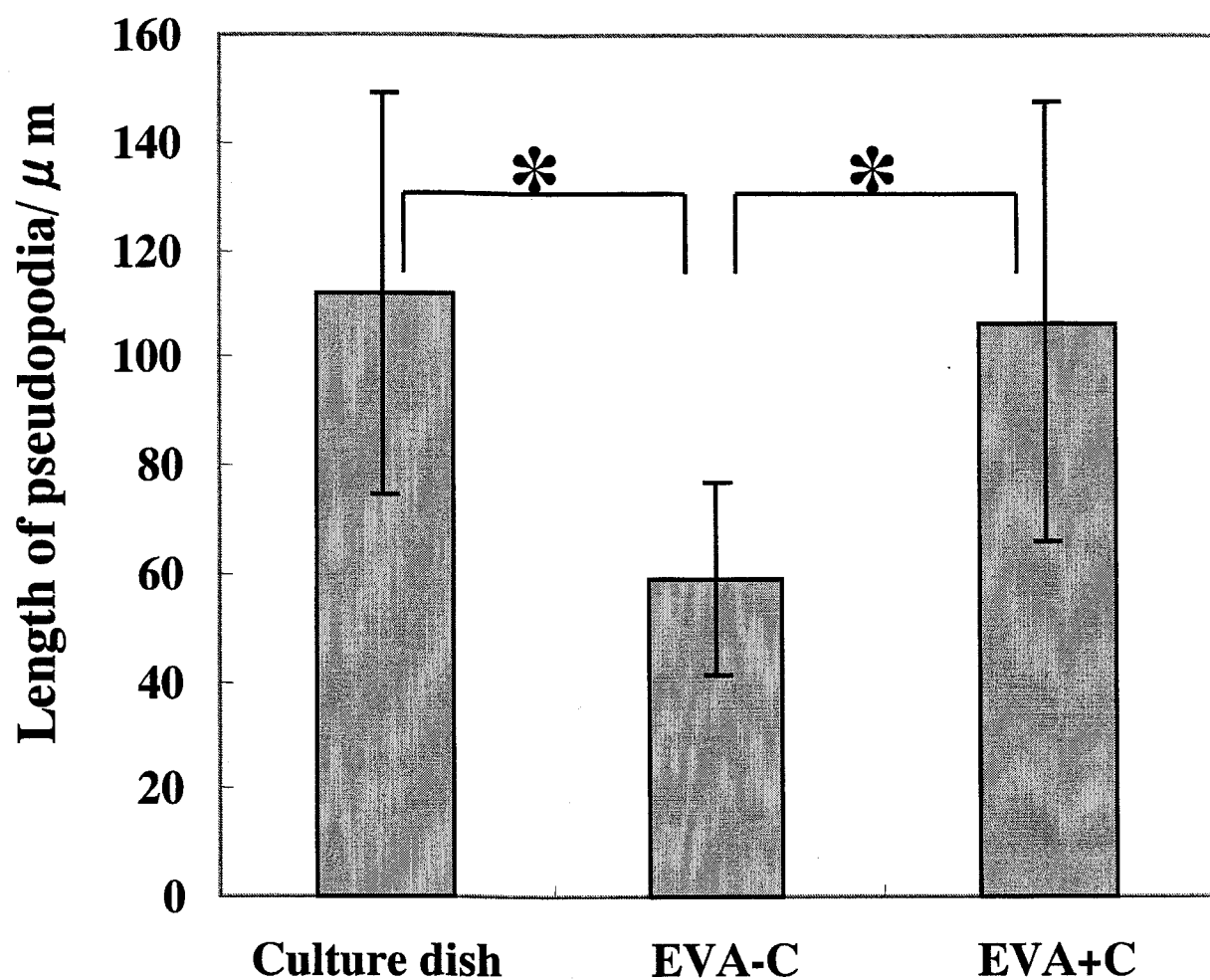


Fig.5 Length of pseudopodia of PDL cells adhered on EVA films after 1 day in the presence of 10 v/v% FCS. Error bar shows S.D. (mean  $\pm$  S.D. n=20).



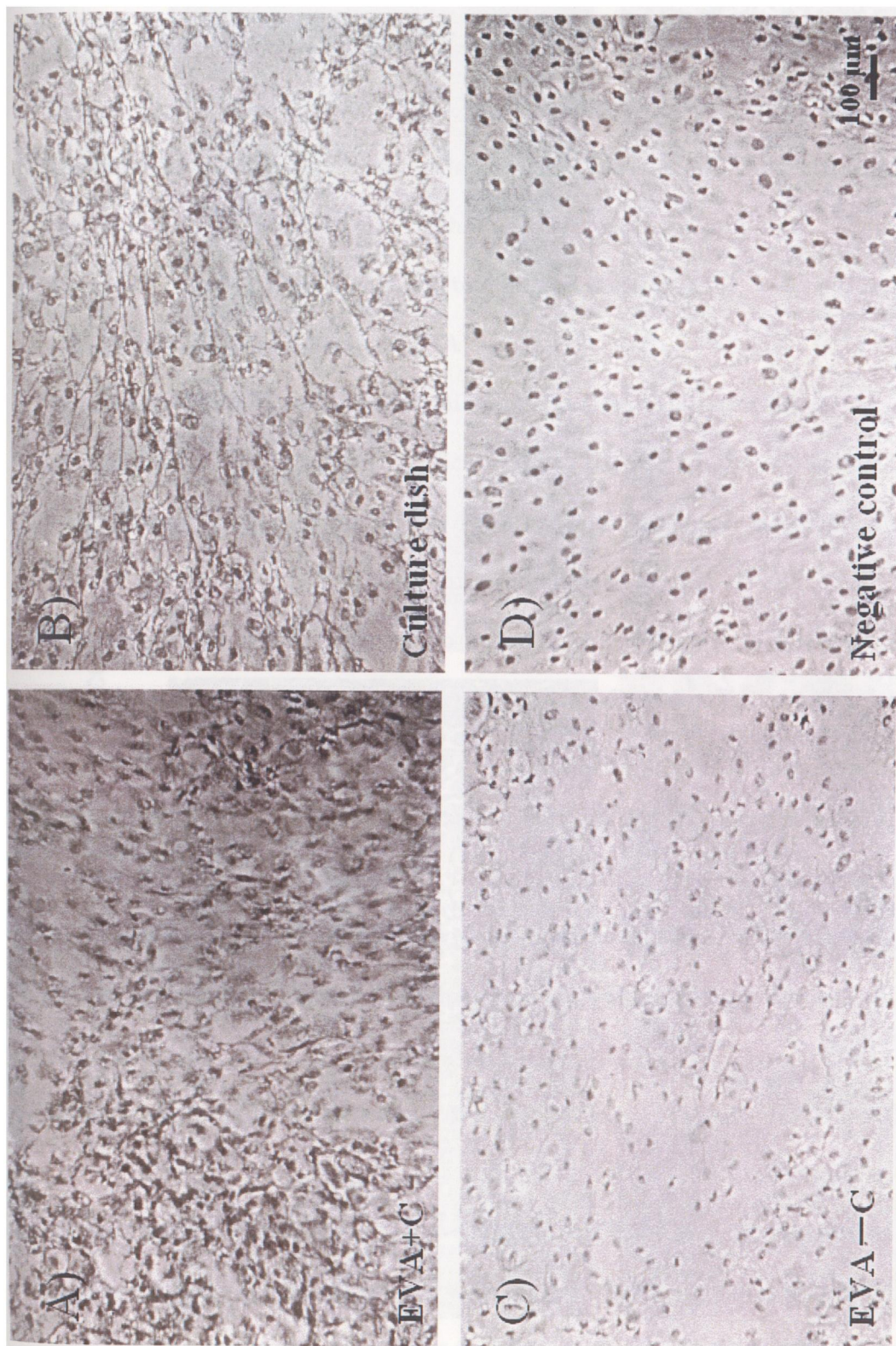


Fig.6 Immunostaining of collagen type I in PDL cells cultured on EVA films for 12 days



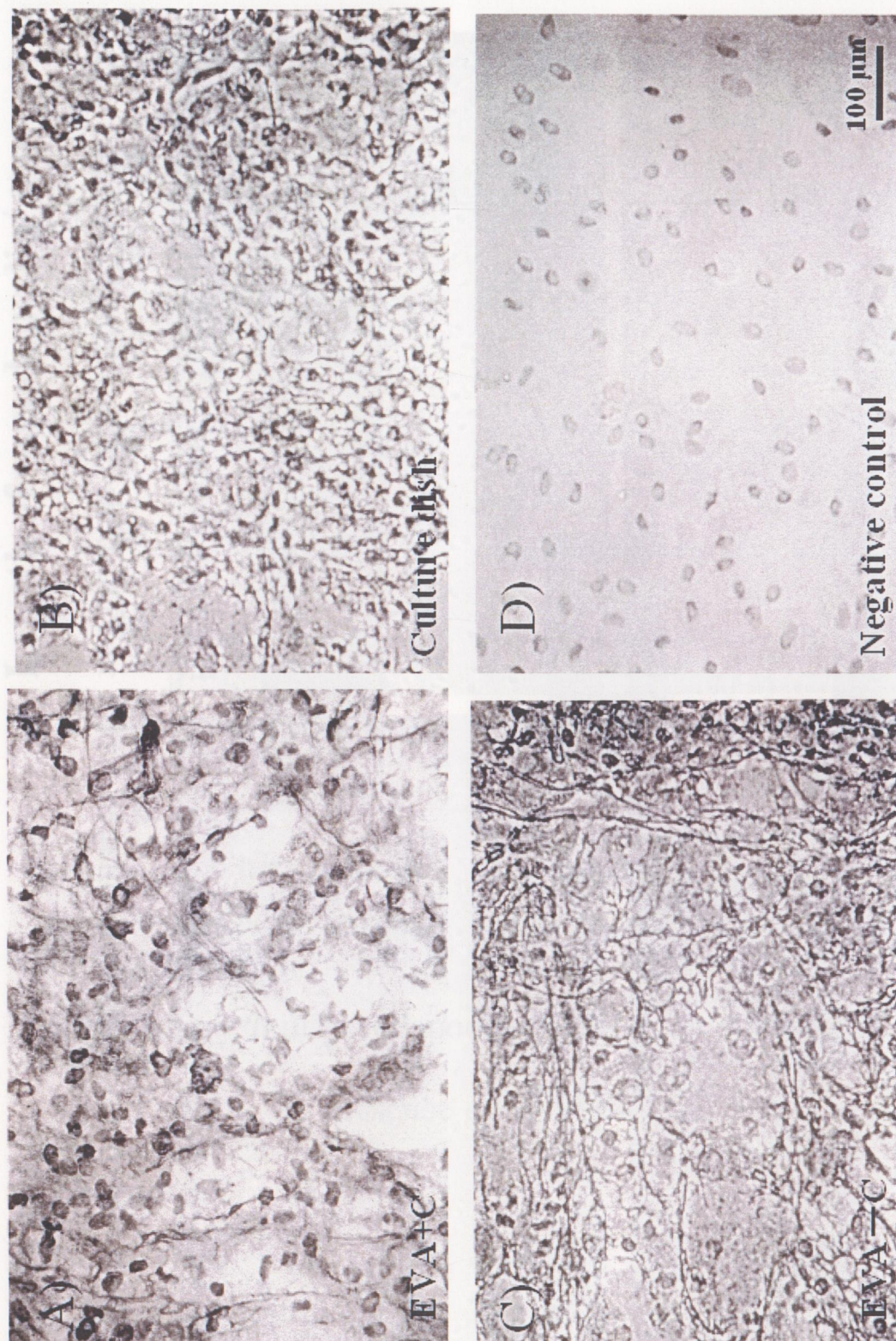


Fig.7 Immunostaining of FN in PDL cells cultured on EVA films for 12 days in the presence of 10 v/v% FCS.

implants to investigate the dynamic effects of dental implant on alveolar bone [10-12]. In order to require the functional recovery of the dental implant, a trial of regenerating PDL around implant was carried out by culturing PDL cell on a novel hybrid implant (titanium+EVA+collagen). The collagen immobilized on EVA surface to enhance the biocompatibility with PDL cells. In chapter 2, the carboxyl groups have been produced on EVA surface by ozone exposure, enabling to immobilize collagen [8].

### **Cell morphology and growth on EVA**

To evaluate the biocompatibility of above materials, cell culture was performed in the present study. Fig.1 shows SEM micrographs PDL cell morphology cultured on EVA films for 3 and 5 days. PDL cells on EVA+C films became flatter and more extended than those on EVA-C after 3 days. In 5 days culture, the pseudopodia of cells became far longer and extended in the same direction. To obtain the insight into cellular adhesion response to EVA+C and EVA-C, the number of PDL cells adhered and the extent of extension were measured. Growth curves of PDL cells on EVA dishes with or without FCS were given in Fig.2. The cell growth on EVA+C dishes showed almost the same profile as that on conventional culture dish in the presence of FCS, though no proliferation was observed on EVA-C ( $p<0.01$ ). When the culture was carried out without FCS (as show in Fig.3), only PDL cells on EVA+C dishes well proliferated ( $p<0.01$ ), which strongly suggested high compatibility of EVA+C with PDL cells.

### **Cell adhesion and extension on EVA**

The PDL cell adhesion and extension on EVA were investigated after 1 day culture with 10 v/v% FCS and the results were given in Fig.4 and 5, respectively. As shown in these figures, both the number of adhesion and the length of pseudopodia of PDL cells on EVA+C were significantly higher than those on EVA-C ( $p<0.01$ ). These results strongly suggested the



importance of collagen for cell adhesion and growth on EVA.

### **Synthesis of type I collagen and FN**

For the purpose of PDL regeneration, the collagen synthesis by PDL is essential. Cell adhesion to the extracellular matrix (ECM) is an also indispensable event in cell growth, differentiation, and migration. FN is one of the fundamental cell adhesion proteins for attachment to ECM. FN synthesis was also detected in this study. Immunostaining microphotographs of PDL cells cultured on EVA against type I collagen and FN were given in Fig.6 and 7, respectively. In this experiment, PBS(-) was used as negative control instead of anti-collagen and anti-FN. Type I collagen secretion was observed in PDL cells on EVA+C and culture dish (Fig.6), although FN synthesis was found in all of the samples (Fig.7).

### **Conclusion**

Human PDL cell culture on EVA indicated the importance of the presence of collagen for cell adhesion and proliferation. Type I collagen and FN production were observed on the EVA+C films. These results suggest that PDL cell culture on hybrid dental implant (Collagen+EVA+Titanium implant) could lead to PDL regeneration around dental implant.

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## **Chapter 4**

### **Surface Modification of EVA; Hydroxyapatite Immobilization and Control of PDL Cells Differentiation**

#### **Introduction**

A periodontal ligament (PDL) lies between the cementum and the alveolar bone and plays quite important roles such as a shock absorber against the mastication impact and receptor of biting forces [1,2]. PDL is the fibrous connective tissue and contains heterogeneous cell populations including the fibroblast, osteoblast, blood vessel cells, nerve cells, and mesenchymal stem cells [3-5]. Generally, the cells including all cells derived from PDL are called PDL cells. It is thought that these PDL cells have an ability to maintain the structure of the periodontal ligament and surrounding alveolar bone and cementum [6]. Usage of a guided tissue membrane for preventing the downgrowth of the gingival tissue is effective to the regeneration of the periodontal tissues in the case of periodontal disease [7,8], which suggests that the PDL cells are differentiated to suitable cell phenotypes in the suitable place such as the cementoblasts in the cementum, fibroblasts in the fibrous tissue, and osteoblast in the alveolar bone. Indeed, it was reported that the PDL has been regarded as a source of cementoblasts and osteoblasts [9]. PDL cells showed osteoblastic differentiation in response to dexamethasone [10,11] and mechanical stress [12]. The bone-like properties were usually characterized by the upregulation of alkaline phosphatase activity [13,14] and osteocalcin mRNA [15,16]. However, the mechanism of PDL cell differentiation to bonelike cells has been still unclear. Previous chapters showed the possibility of development of regeneration of PDL around an artificial implant by culturing PDL cells [17-19]. Briefly, this

hybrid implant is explained as follows: Poly(ethylene-co-vinyl alcohol)(EVA) was coated on a titanium implant [17]. The adhesive strength between EVA and titanium was very high (about 15MPa) due to the electrostatic force and the water resistance was higher than other adhesives. Biocompatibility can be enhanced by collagen immobilization onto the EVA surfaces [18,19]. An additional aim of this study is to control the PDL cell differentiation and to construct the highly organized hybrid implant, and to regenerate the periodontal tissues including cementum, PDL, and alveolar bone. Generally, periodontal ligament fibers comes into the cementum or alveolar bone, which makes the tooth root strongly fixed. Cementum was the highly calcified tissue and metabolized by cementoblast and cementoclast. The cementum and bone are very resembled each other and still unclearness in the difference. Cementoblasts also show bonelike cells properties such as high alkaline phosphatase activity and osteocalcin secretion [20]. Osteocalcin is also one of the most well-characterized marker of calcified tissues [21].

Many investigations [22-24] have been carried out to develop hydroxyapatite coating onto orthopaedic and dental materials to promote rapid attachment to bone. Various techniques such as plasma spray [25], ion-beam-assisted deposition [26], sol-gel processing [22], have been used for coating. In this chapter, the author chose alternate soaking method [27] to coat hydroxyapatite on an implant because of its easiness and high efficiency for modification.

## **Materials and methods**

### **Materials**

EVA ( $\overline{M}_n = 15,000$ , 200  $\mu\text{m}$  in thickness) having 56 mol% of vinyl alcohol content was kindly supplied by Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan). These films were cut into 10 or 20 mm diameter and stored in acetone at 25 °C until usage. One

w/w % type I collagen solution (pH 3.7, in 5 mM phosphoric acid) was kindly donated by Nitta Gelatin Co. Ltd. (Osaka, Japan). All other materials were of chemical reagent grade and used without further purification.

### **Sample preparation**

#### **Carboxyl groups introduction on EVA**

In order to convert hydroxyl groups of EVA into carboxyl groups, EVA films were exposed to ozone as described in chapter 2 [18]. In brief, ozone gas was produced by a generator (POX-10, Fuji Electric Co., Ltd, Tokyo, Japan) equipped with an O<sub>2</sub> cylinder and taken into a glass bottle in a water bath at 70°C at a rate of 1.0 l/min. EVA films were hung in the bottle and treated with 0.5 g/h of ozone gas flow for 5h. Then 0.1 μmol/cm<sup>2</sup> of carboxyl groups were introduced on the EVA surface (COOH-EVA), evaluated by simple neutralization titration.

#### **Collagen immobilization on the EVA**

Chapter 2 shows details of preparation of the collagen immobilized EVA (COL-EVA) surfaces were reported [18]. EVA films having carboxyl groups were immersed in 0.5 w/w% type I collagen solution (pH 3.7, in 5mM phosphoric acid) and gently stirred at 25 °C for 16 h. After the formation of a polyioncomplex, unimmobilized collagen was removed by rinsing the films with an excess amount of 5mM of phosphoric acid (pH 3.7). The amount of collagen immobilized on the EVA surface was 6 μg/cm<sup>2</sup>, evaluated by the ninhydrin method [28].

#### **Hydroxyapatite immobilization on the EVA**

The EVA films having carboxyl groups were soaked in 200mM CaCl<sub>2</sub> / 50mM Tris(tris hydroxymethyl aminomethane)-HCl (pH 7.4) solution at 25°C for 12h. And the films were subsequently soaked in 120mM aqueous Na<sub>2</sub>HPO<sub>4</sub> solution at 25°C for 12h. This alternate immersion was repeated and continued for 10 times. After each cycle of the alternate

immersion, the hydroxyapatite immobilized EVA (HAP-EVA) films were washed by ultrasonic wave in distilled water to remove the physisorbed hydroxyapatite.

#### **Preparation of hydroxyapatite and $\beta$ -tricalcium phosphate plate**

Hydroxyapatite(HAP) and  $\beta$ -tricalcium phosphate( $\beta$ -TCP) plates were also prepared. Pure powder of HAP and  $\beta$ -TCP was sintered and molded to disks with 20 mm in diameter at 1200 and 1150 °C for 2 h, respectively. Light microscopic photographs of HAP,  $\beta$ -TCP and HAP-EVA were given in Fig1, whose surfaces were relatively smooth and having almost the same roughness.

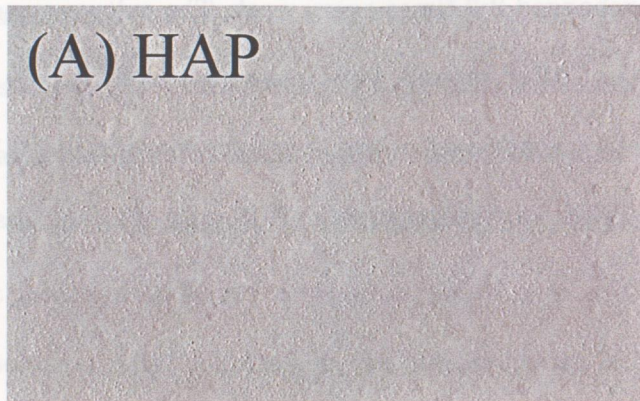
#### **X-ray diffraction**

Hydroxyapatite immobilized was characterized and compared with the pure HAP and  $\beta$ -TCP using an X-ray diffractometer (Philips X'pert-MPD, Koninklijke Philips Electronics N.V., Netherlands). CuK $\alpha$  radiation was used for the diffraction with a voltage of 40kV and a current of 55mA.

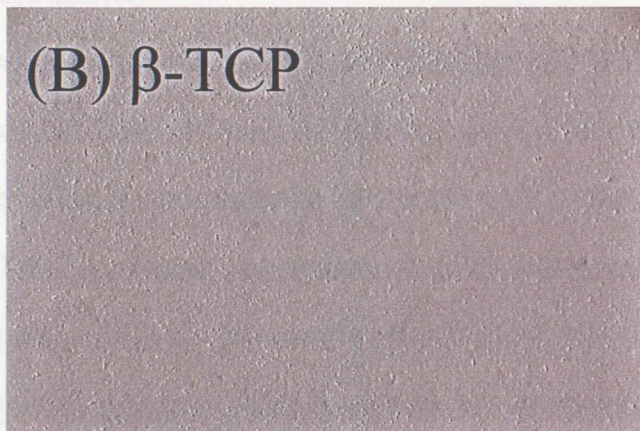
#### **Cell culture**

A human healthy third molar extracted by orthodontic reasons was stored in phosphate buffer solution (PBS(-)) containing 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin, and 50  $\mu$ g/ml gentamycin at 4 °C immediately after extraction. The periodontal ligament attached to the mid-third of the root was minced (1-3 mm<sup>2</sup>) and recovered with a scalpel to avoid contamination by gingival and pulpal cells. The periodontal ligament tissue was placed in a tissue culture dish with 0.2 ml of Dulbecco's modified Eagle's medium (DMEM)(supplemented with 10 v/v% of fetal calf serum, 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin, and 50  $\mu$ g/ml gentamycin), followed by putting a cover glass on the tissue. At the beginning of the culture, 2-3 droplets of DMEM were put into a side of the cover glass.

(A) HAP



(B)  $\beta$ -TCP



(C) HAP-EVA

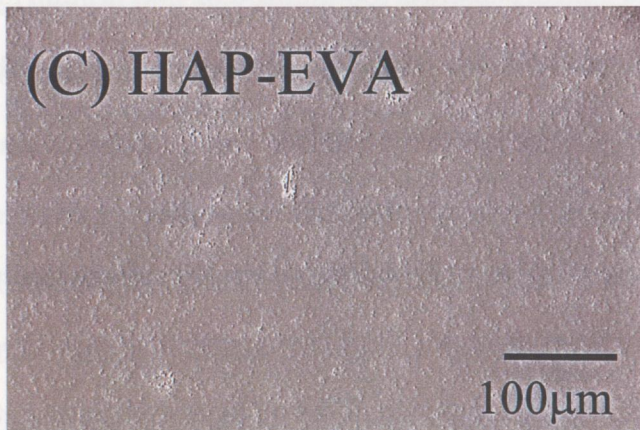


Fig.1 Light microscopic photographs of (A) HAP, (B)  $\beta$ -TCP and (C) HAP-EVA surfaces.



Culture medium was exchanged 2-3 times every week. The cultures were kept at 37°C in a incubator of 5v/v% CO<sub>2</sub>. After several days, migration of PDL cells on culture dish was observed. When the cells reached confluence, they were recovered by 0.1 w/v% of trypsin and 0.025 w/v% of EDTA in PBS(-) and subcultured. PDL cells of 2-5th passage were utilized for this study.

### **PDL cell growth**

To evaluate PDL cell proliferation in the different surfaces, cells were seeded onto the films of COOH-EVA, COL-EVA, and HAP-EVA, plates of HAP and  $\beta$ -TCP, and tissue culture dishes with 20mm in diameter at the concentration of 2500 cells/cm<sup>2</sup> with 2 ml of DMEM. Cell number was evaluated by the lactate dehydrogenase (LDH) activity [29], where LDH oxidizes reduced nicotinamide adenine dinucleotide (NADH) to its oxidized form and the amount of LDH is a function of cell number. Briefly, 3 ml of cell lysate containing 2 v/v% Triton X-100 /PBS(-) and 1 ml of substrate containing 0.62 mM lithium pyruvate and 0.18 mM NADH in 50mM phosphoric acid was mixed and the decrease in the absorbance at 340 nm from 30 sec to 60sec just after mixing was recorded.

### **Calcium determination**

The calcium concentration on EVA films and in the culture medium was measured by an o-cresolphthalein complexon method [30]. Hydroxyapatite immobilized EVA film ( $\phi$ 10mm) was put into 1ml of 0.1N HCl in a test tube, followed by hydrolyzation at 120°C for 1h. Three ml of 0.88M monoethanolamine buffer (pH11.0) and 0.5ml of color developing reagent (containing 0.63 mM o-cresolphthalein complexon and 69mM of 8-hydroxyquinoline) were added to the solution and the color development was recorded at 570 nm. In the case of the culture medium, 100  $\mu$ l of medium was added to the 3ml of buffer and 0.5 ml of color reagent.

### **Phosphorus determination**

The phosphorus concentration on EVA films was measured by a molybdenum blue method [31]. The same manner was employed for hydrolyzation as mentioned above. Three ml of the reduction solution (containing 77mM ammonium ferrous sulfate) and 0.5ml of color developing reagent (containing 36mM ammonium molybdate and 102mg/ml sulfuric acid) were added to the solution. After colorization, the absorbance at 750 nm was measured.

### **Alkaline phosphatase activity**

PDL cells were seeded onto COOH-EVA films, COL-EVA films, HAP-EVA films, HAP-plates,  $\beta$ -TCP plates, and conventional tissue culture dishes with 20mm in diameter at a concentration of 8000 cells/cm<sup>2</sup> with 2 ml of DMEM and cultured. Alkaline phosphatase activity of the PDL cells at confluent state was evaluated by p-nitrophenyl phosphate method [32], 3 ml of cell lysate (containing 2 v/v% Triton X-100 /PBS(-)) and 0.5 ml of substrate solution(containing 2 mM MgCl<sub>2</sub> and 33 mM disodium p-nitrophenyl phosphate (pH9.8)) was added. After colorization at 37 °C for 15min, the reaction was stopped by adding 0.5ml of 2N NaOH and the absorbance at 405 nm was measured.

### **Enzyme linked immunosorbent assay (ELISA) for osteocalcin**

After 7 or 14 days culture of PDL cells on the different surfaces, medium were changed to 1ml of serum free medium and further culture was proceeded for 48 h. The concentration of the secreted osteocalcin in the culture medium was measured by a commercially available enzyme linked immunosorbent assay kit (osteocalcin ELISA kit, Takara Bio Inc., Shiga, Japan) employing highly specific monoclonal antibody. Each well of 96well plate on which anti-osteocalcin was immobilized was filled with 100  $\mu$ l of culture medium at 25 °C for 1h. After washing 3 times with PBS(-), 100  $\mu$ l of 1 $\mu$ g/ml peroxidase-labeled anti-osteocalcin was added and incubated at 25 °C for 1h. Following 4

times washing, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine solution was added and the absorbance at 405 nm was measured by a plate reader (versa max, Molecular Devices Co., CA. USA).

## Results and discussion

### Hydroxyapatite immobilization onto EVA

Fig.2 shows that the adsorption of calcium ions on the carboxyl groups of EVA depended on the amounts of carboxyl groups and the calcium concentration. Indeed, no carboxyl groups on EVA showed no adsorption of calcium, indicating that the adsorption of calcium ions was dominated by the chemical equilibrium such as chelate formation between  $\text{Ca}^{2+}$  and  $\text{COO}^-$ . The amount of the calcium and the phosphorus adsorbed on the EVA by the alternate soaking was given in the Fig.3 and 4, respectively. Both the amount of calcium and phosphorus increased with an increase in the alternate soaking times and the amount of the carboxyl groups. Beyond 4 times soaking, a thin layer of calcium deposition was observed on the EVA surface. The hydroxyapatite crystal formation was multiplied during this cycle. After 10 times, a lot of small crystals on the film become completely white. It is likely that the difference in the initial concentration of carboxyl groups on EVA gave the difference in the amount of calcium adsorption (Fig.2), which affected the total amount of calcium and phosphorus during this cycle. In fact, no carboxyl groups made no hydroxyapatite deposition (Fig.3 and 4). Table 1 shows the molar ratio of the calcium to phosphorus and the ratio was around 1.6, regardless of the differences in COOH amount and cycle number, lower than that of pure hydroxyapatite (1.67). So the immobilized calcium phosphate consisted of various calcium phosphates such as hydroxyapatite, tricalcium

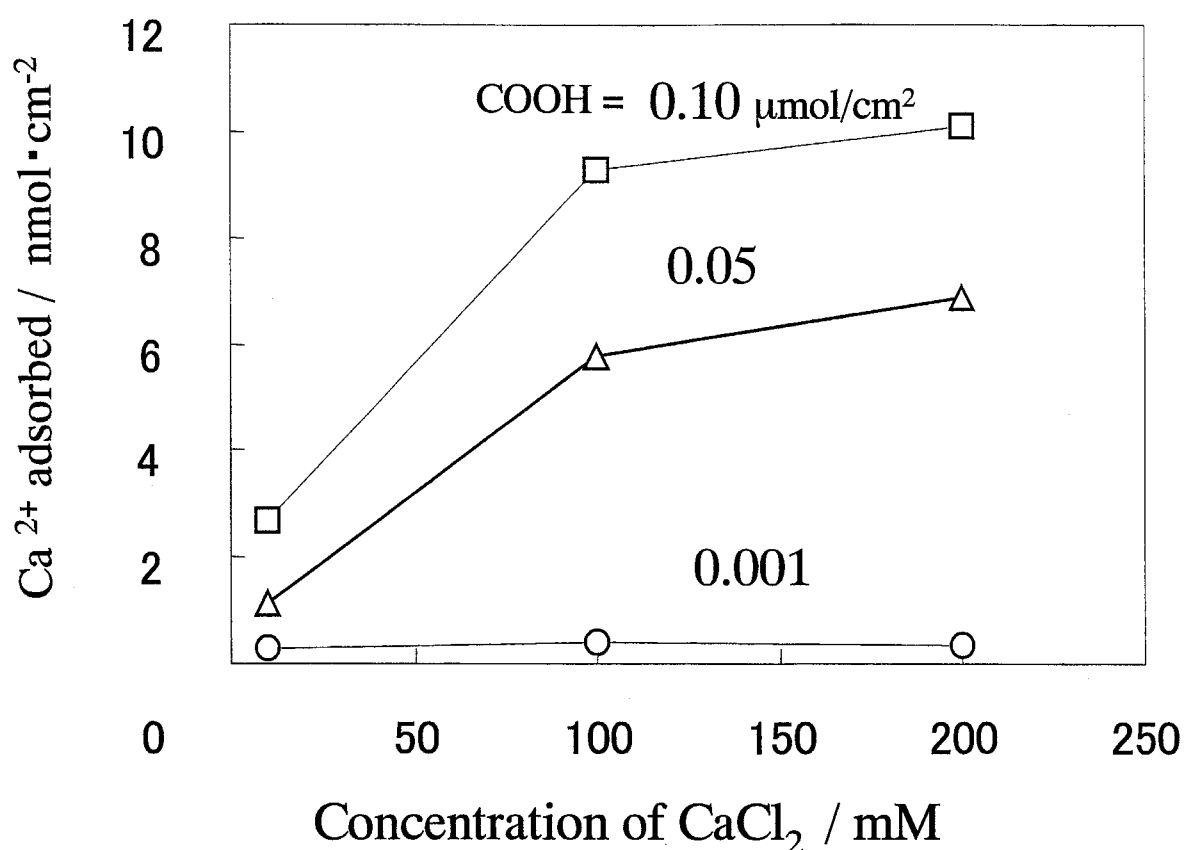


Fig.2 Calcium ions adsorbed onto COOH-EVA surface after immersion in different concentration of calcium at pH 7.4 and 25 °C for 12 h.

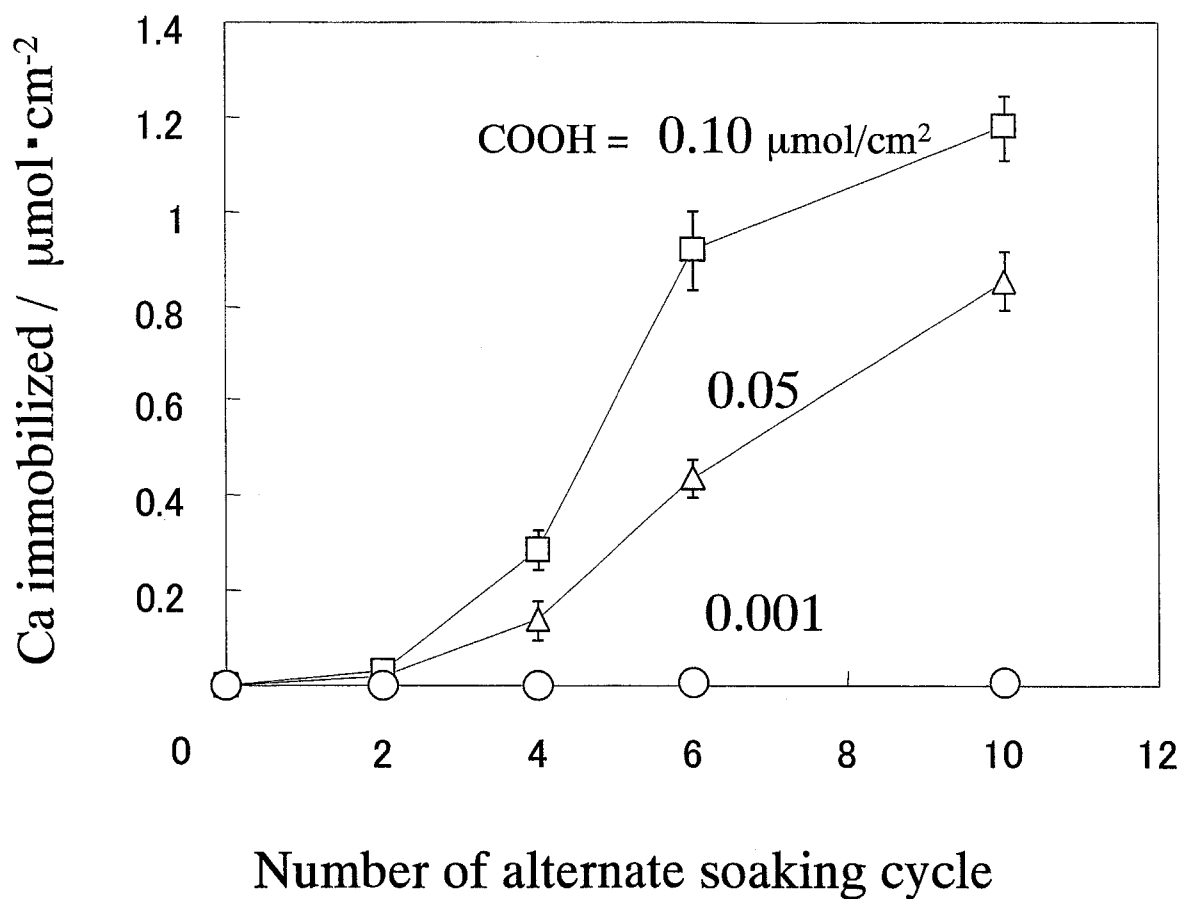


Fig.3 Relationship between alternate soaking cycle and calcium immobilization on COOH-EVA with various amount of carboxyl groups. The error bars indicate the SD (n=3).

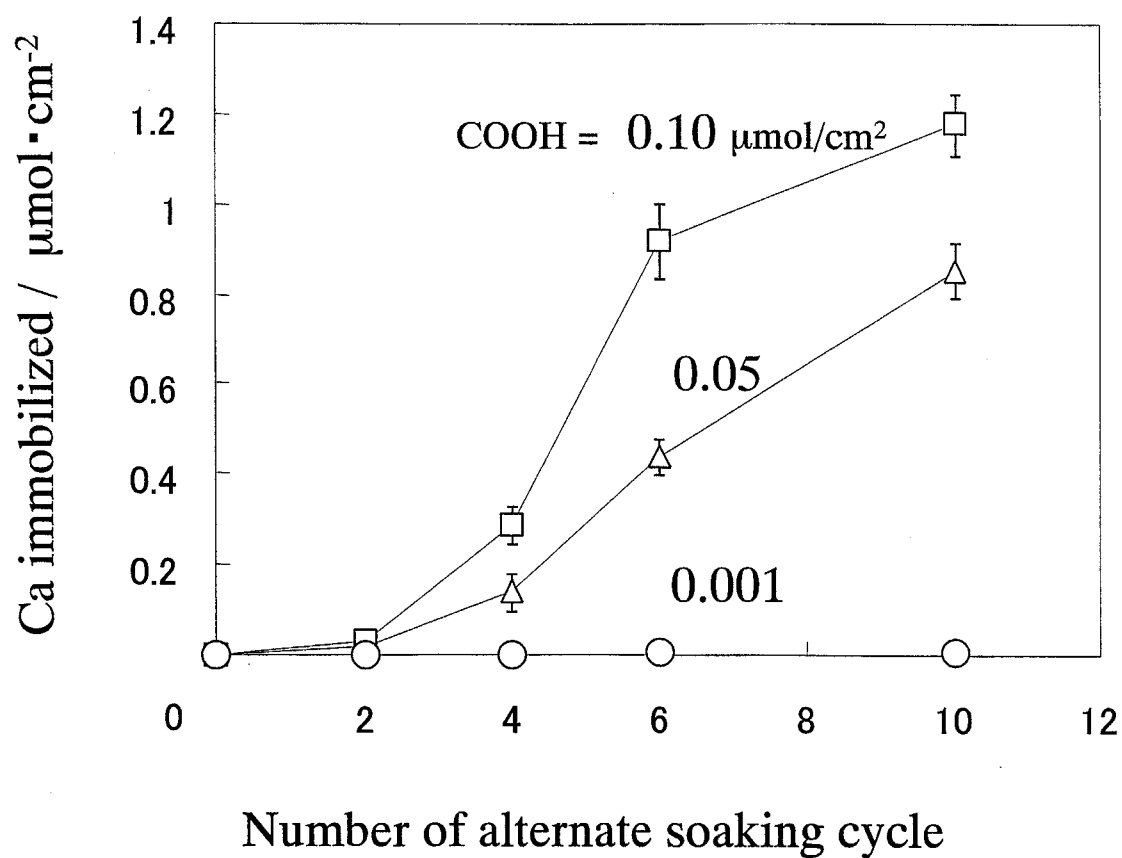


Fig.4 Relationship between alternate soaking cycle and phosphorous immobilization on COOH-EVA with various amount of carboxyl groups. The error bars indicate the SD (n=3).

phosphate or carbonate apatite due to the carbon dioxide in the air [33]. In the alternate soaking method, it was thought that the crystals formation was considerably abrupt and the small and various crystallization and composition of calcium phosphate were formed. Fig.5 shows the X-ray diffraction pattern of the deposition powder scraped off from the surface of the alternate soaked EVA films and the pure hydroxyapatite and  $\beta$ -TCP plate. The crystallinity and the amount of the crystal of the deposits were increased with an increased in the cycle number and the deposited crystals were almost the same as hydroxyapatite having low crystallization [27].

#### **Cell proliferation**

Proliferation of PDL cells on various substrates was given in Fig.6. PDL cells showed highest proliferation on the COL-EVA. On the other hand, on the HAP-EVA and HAP, cell growth was slightly inhibited rather than on other surfaces. Chapter 3 suggested that biocompatibility of EVA was markedly enhanced by collagen immobilization [19]. Collagen is known to be biocompatible material and used to the scaffold of the cells and growth factors on the tissue engineering [34]. Fig.6 gives the doubling time of the PDL cell was 39.2 (COL-EVA), 41.4 (tissue culture dish), 42.1 (COOH-EVA), 51.2( $\beta$ -TCP), 52.9 (HAP-EVA), and 56.4 h (HAP). HAP is well-known and applied in the orthopedic and dental implants for direct connection and fixation between bone and implant surface [23,24] and shows no cytotoxicity. However, growth rates of PDL cells on the HAP,  $\beta$ -TCP, HAP-EVA were lower than those on COL-EVA and COOH-EVA suggesting the cell differentiation on HAP.

#### **Change in calcium concentration on different surfaces**

Fig.7 showed the change in calcium concentration in the absence of PDL cells. Calcium ions in the medium on HAP, HAP-EVA, and  $\beta$ -TCP decreased compared with tissue

Table 1

The molar ratio of calcium to phosphorous in the crystallites immobilized on COOH-EVA

COOH on EVA/ $\mu\text{mol} \cdot \text{cm}^{-2}$	0.05				0.10			
Cycle number / time	2	4	6	10	2	4	6	10
Ca/P	1.50	1.64	1.60	1.57	1.60	1.60	1.51	1.62

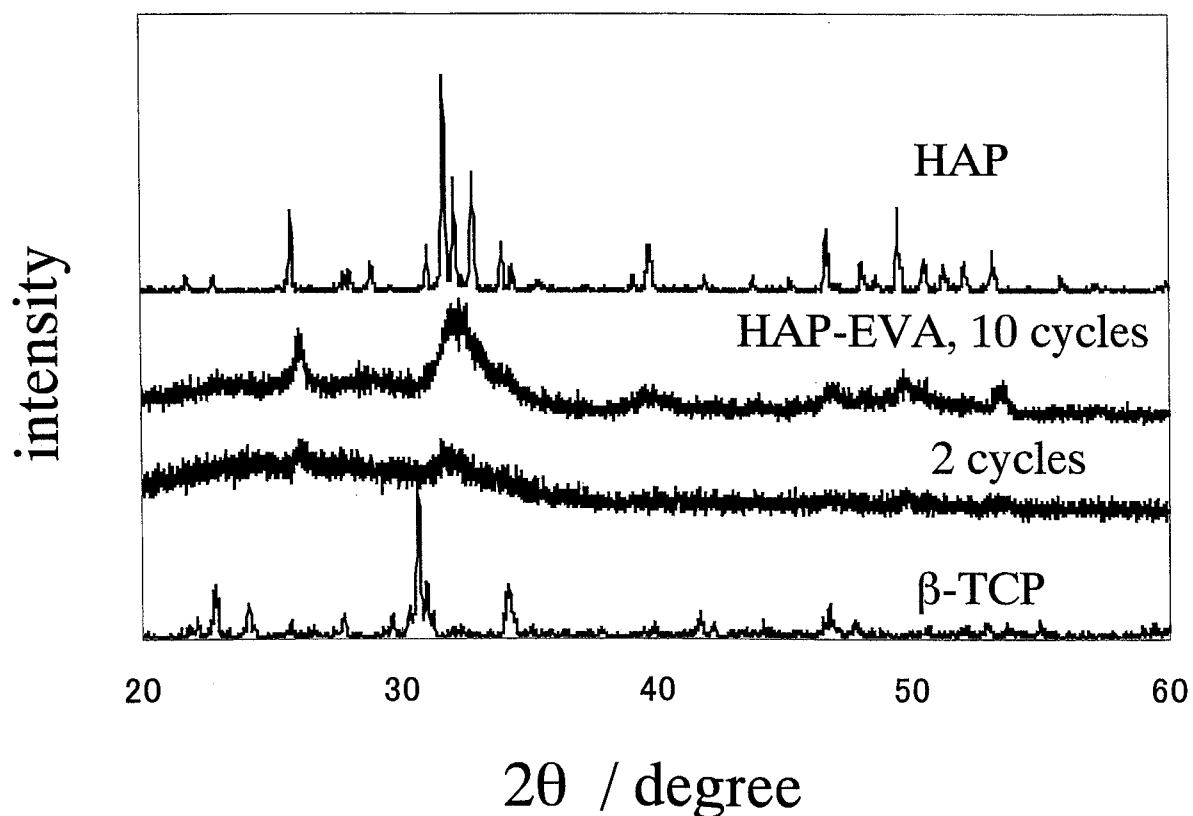


Fig.5 X-ray diffraction pattern of the HAP immobilized on EVA by alternate soaking, surface of pure HAP and  $\beta$ -TCP plates.



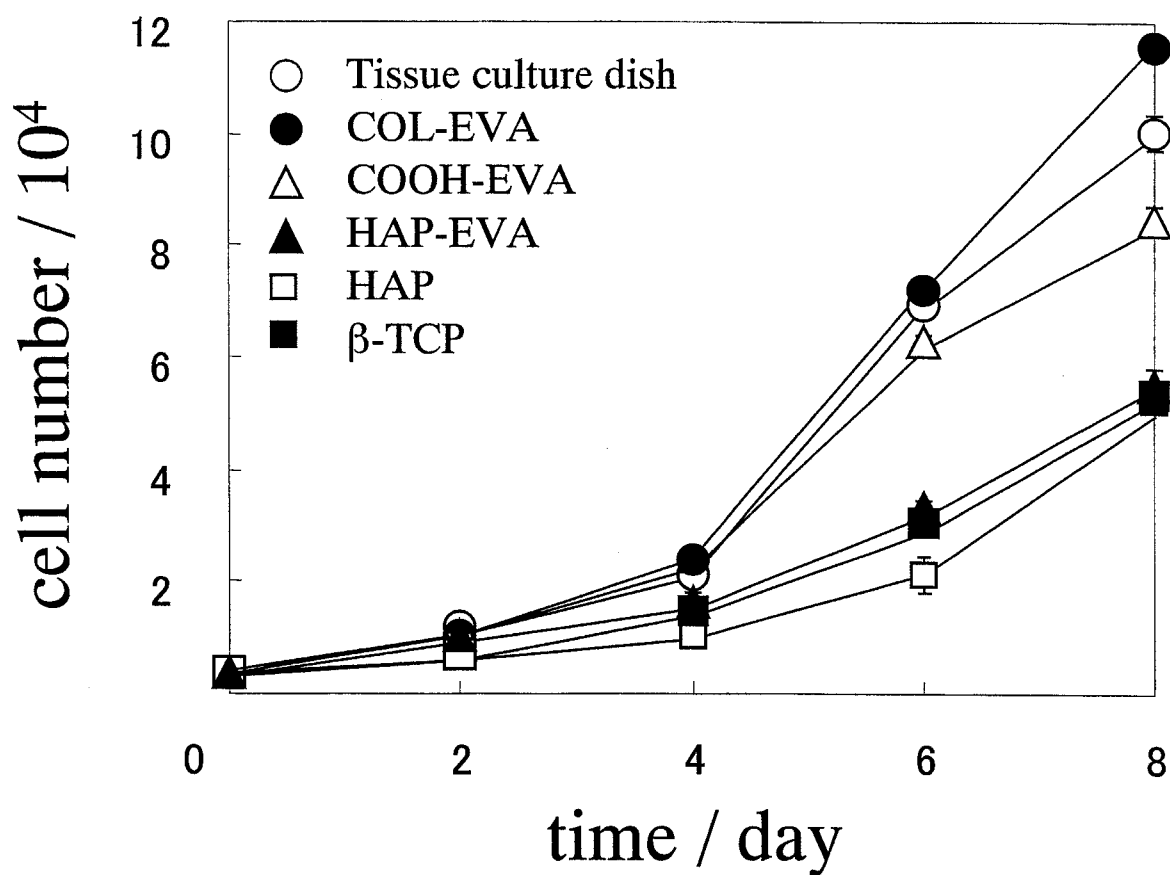


Fig.6 PDL cell growth on the various surfaces. The error bars indicate the SD (n=3).

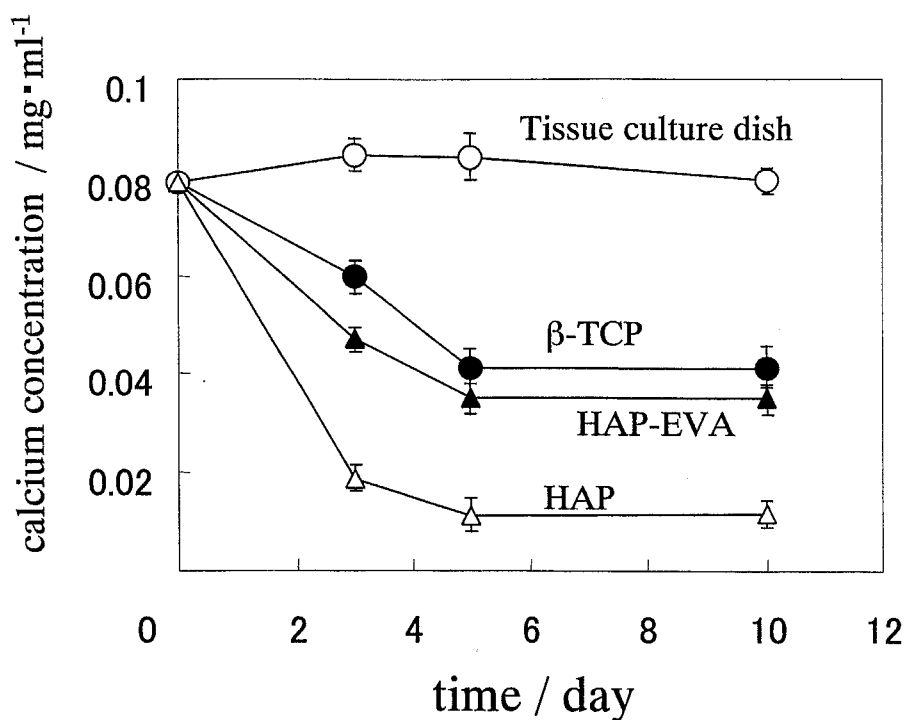


Fig.7 Calcium ion concentration in the medium on various materials without cells. The error bars indicate the SD (n=3).

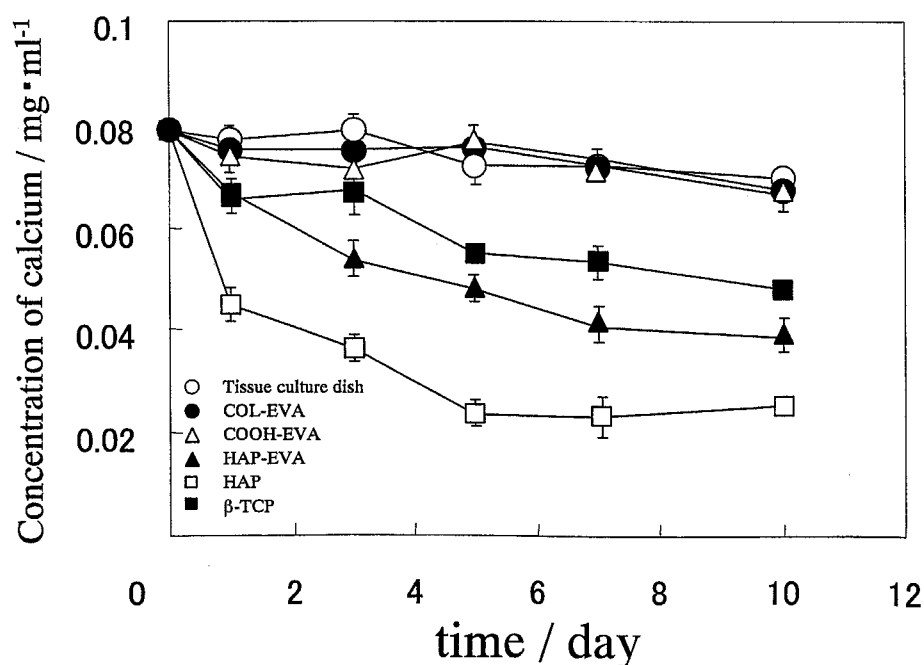


Fig.8 Calcium ion concentration in the medium on various materials in the presence of cells. The error bars indicate the SD (n=3).

culture dish, suggesting that the new crystallites of calcium phosphate were precipitated on the surface [23]. In contrast, calcium change in the presence of PDL cells was given in Fig.8. PDL cells made no change in calcium concentration on tissue culture dish, COL-EVA and COOH-EVA. The decrease profiles on HAP, HAP-EVA and  $\beta$ -TCP with cells was almost the same as that of without cells. However, the amount of reduction of calcium ion in HAP and  $\beta$ -TCP was much lower than that of without cells. And almost same reduction was observed in HAP-EVA. From the result of Fig.7, the calcium ion concentration did not change from 5 to 10 days due to the chemical equilibrium of precipitation of crystallites. In contrast, from Fig.8, the reduction of calcium concentration was continued after 10 days especially on HAP-EVA and  $\beta$ -TCP, suggesting that the cells on these surfaces took up calcium ion and actively precipitated onto the surface. The interface between hydroxyapatite and EVA was bound strongly by electrostatical force not to wash away by ultrasonic treatment. Titanium, EVA, and hydroxyapatite were electrostatically adhered and on which the cells can metabolized and deposit the new cementum like matrix.

#### **Alkaline phosphatase activity**

Alkaline phosphatase activity of PDL cells cultured on the different surfaces at 7 and 14 days is given in the Fig. 9, where data were normalized not by total protein amount but by the cell number. Alkaline phosphatase is a glycoprotein, mineralizing in the calcified tissue such as bone and cementum [35]. At 7 days culture, no significant difference in alkaline phosphatase activity was observed. The activity was the highest on the HAP-plate and lowest on the COOH-EVA at 14 days and HAP-EVA showed no statistically difference against HAP-plate. In this experiment, cells were seeded on the materials to be confluent in 7 days and no proliferation occurred from 7 to 14 days, suggesting the dominant of differentiation.

### **Osteocalcin secretion**

Osteocalcin secretion by PDL cells cultured on the different surfaces for 9 and 16 days were given in Fig.10. The profile was similar to the results in alkaline phosphatase (Fig.9) and the difference was clear after 16 days culture. HAP-EVA showed significant increase in osteocalcin secretion compared with COL-EVA, COOH-EVA, and tissue culture dish, similar to HAP and  $\beta$ -TCP. Osteocalcin is the most abundant noncollagenous protein localized in mineralized tissue and plays an important role in bone remodeling. This calcium binding protein represents the peculiar biochemical marker of final differentiation of osteoblast and produced by also odontoblast and cementoblast. A number of studies reported that osteoblasts are influenced by HAP and  $\beta$ -TCP [23,36]. In the present work, effect of HAP-EVA on PDL cells was compared with the HAP and  $\beta$ -TCP plate, which might be effective on the osteoblastic phenotype, and the differentiation ability of PDL cells was also evaluated. It was reported that the titania / hydroxyapatite composite made mature osteoblast-like cell MG63 raise the osteocalcin secretion ability [23]. Fig.9 and 10 suggest that both alkaline phosphatase activity and osteocalcin secretion increase especially on HAP-EVA, HAP, and  $\beta$ -TCP without cell proliferation after 7 days. In addition, the similar level of these markers on HAP-EVA to those on HAP and  $\beta$ -TCP shows no requirement in high crystallinity in calcium deposition for PDL cell differentiation.

### **Conclusion**

Hydroxyapatite crystalline could be immobilized on EVA films through ozone treatment and alternate immerse method. The PDL cell growth on the HAP-EVA was lower than those on COL-EVA, COOH-EVA, and tissue culture dish. Decrease in calcium concentration in culture medium and increment in peculiar osteoblast markers such as alkaline phosphatase activity and osteocalcin expression suggest that HAP-EVA might

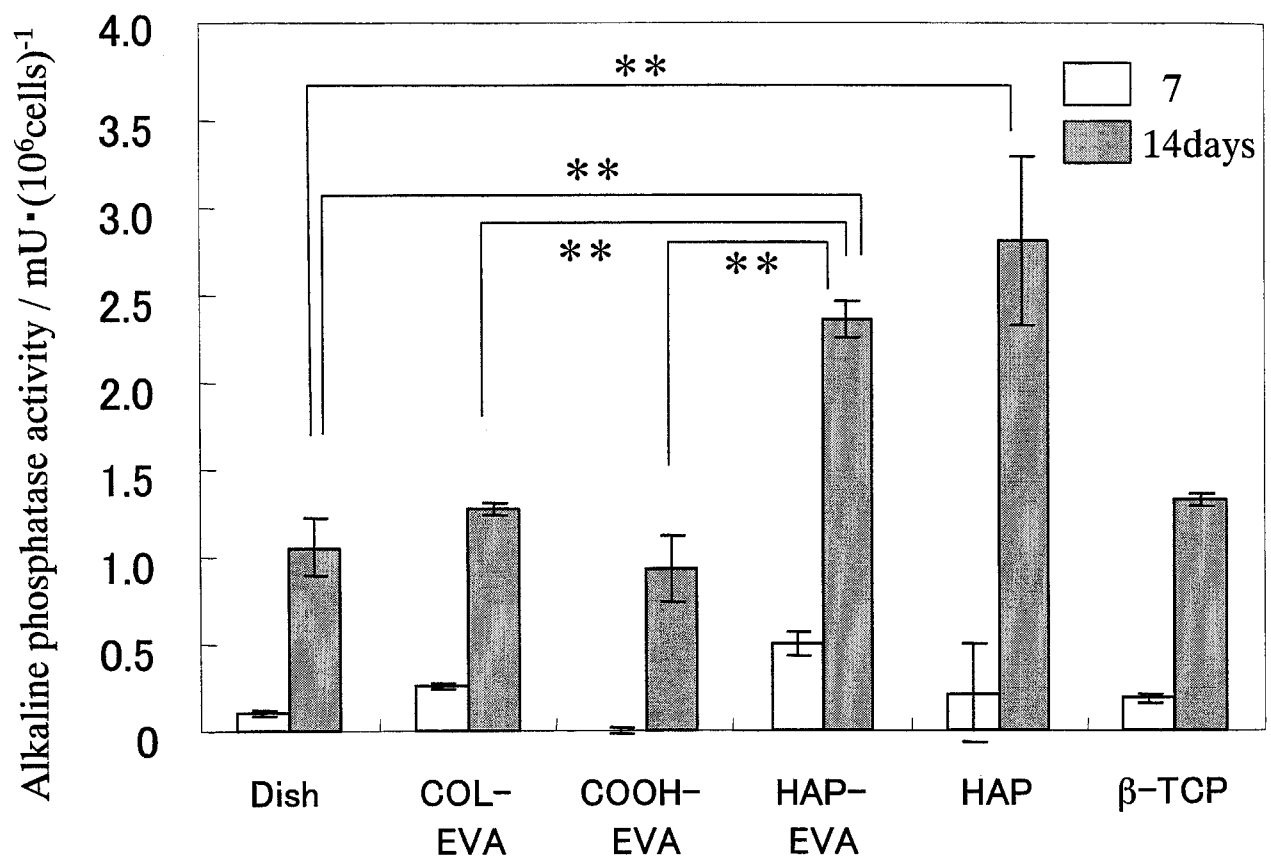


Fig.9 Alkaline phosphatase activity of PDL cells on the various surfaces. The error bars indicate the SD (n=3). \* \* p<0.05

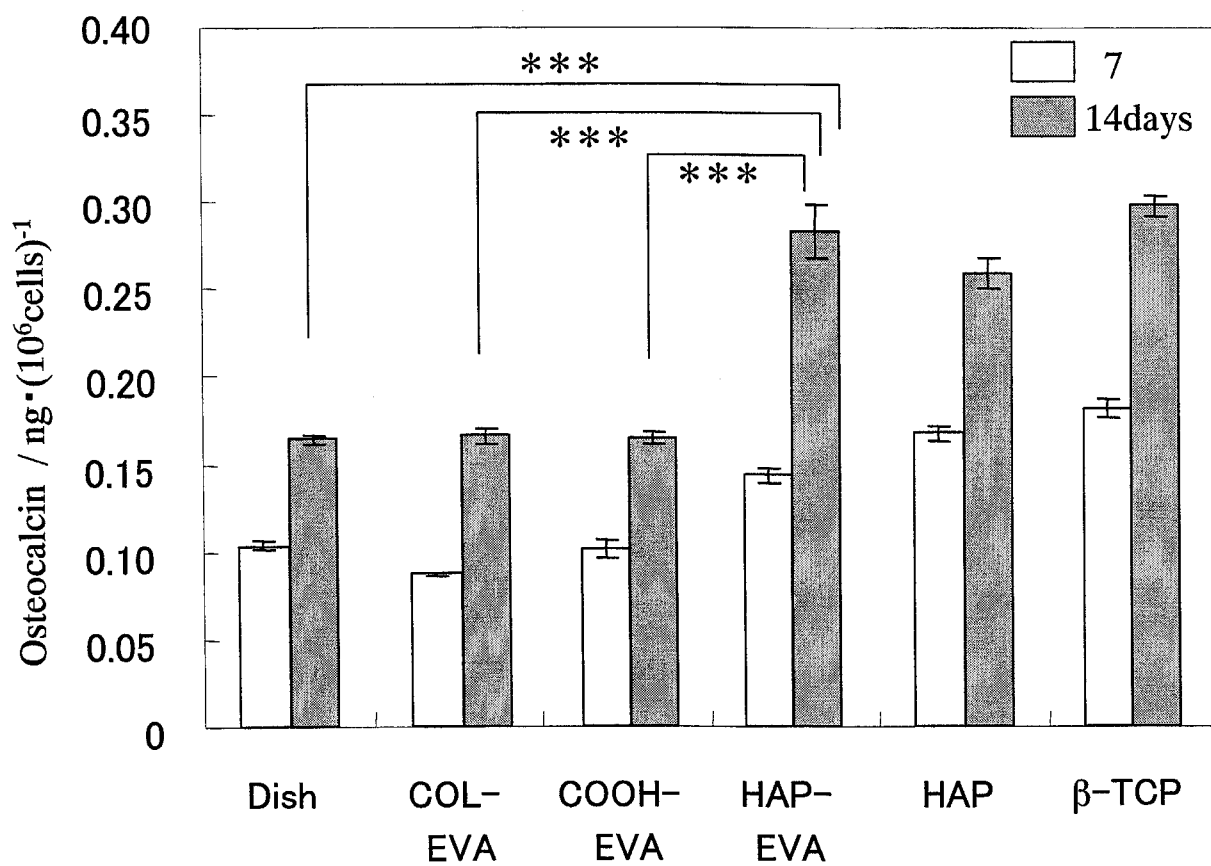


Fig.10 Osteocalcin secretion of PDL cells on the various surfaces. The error bars indicate the SD (n=8). \* \* \* p<0.001

stimulate PDL cells to differentiate to osteoblastic cell like as HAP and  $\beta$ -TCP. Therefore, it was summarized that the differentiation of the PDL cells to mineralized tissue cells could be realized on HAP-EVA, which might make it possible to prepare further highly organized hybrid implant possessing PDL and cementum on the surface of artificial dental implant.

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## **Chapter 5**

### **Differentiation of Gingival Cells by Culturing on Hydroxyapatite Immobilized EVA**

#### **Introduction**

Fibroblasts are major cell type in the connective tissue such as a periodontium [1] and play important roles during development and regeneration. Periodontium includes two kinds of fibroblast, gingival cell and periodontal ligament (PDL) cell [2]. It is likely that gingival cell produces and maintains the soft tissue like mucosa as a simple task, in contrast, PDL cell has a function of producing and maintenance of periodontal ligament, cementum, and alveolar bone [3,4]. In spite of their widely anatomical distribution and many studies on these fibroblasts, they have not been well identified but classified only on the bases of dubious attribute such as spindle shape morphology and their origin [5]. PDL lies between the cementum and the alveolar bone and plays quite important roles such as a shock absorber against the mastication impact and receptor of biting forces [6]. Author has been studied about the regeneration of PDL around artificial implant by culturing PDL cells. Briefly, the hybrid implant is explained as follows: Poly(ethylene-co-vinyl alcohol)(EVA) was coated on a conventional titanium implant [7]. Biocompatibility of EVA can be enhanced by collagen immobilization onto its surfaces [8,9]. PDL cells are seeded onto the modified surface and implanted into alveolar bone to regenerate the periodontium around implant including cementum. In order to achieve this purpose, the property of culturing PDL cells was investigated and differentiation was evaluated. It was confirmed that PDL possesses an ability to regenerate the periodontium and to differentiate toward osteoblast-like cell when cultured on the hydroxyapatite coating EVA surface [10]. These PDL cells have an ability to

maintain the structure of the periodontal ligament and surrounding alveolar bone and cementum. Cementum was the highly calcified tissue and metabolized by cementoblast and cementoclast. The cementum and bone are very similar to each other and still unclearness in the difference. Cementoblasts also show bonelike cells properties such as high alkaline phosphatase activity and osteocalcin secretion [11]. Osteocalcin is also one of the most well-characterized marker of calcified tissues [12] and positive secretion was found in also PDL cells [10]. In addition, PDL has been regarded as a source of cementoblasts and osteoblasts [13]. When it will apply to the hybrid implant, however, large number of PDL cells were needed, which cannot be easily accessible.

Gingival cell doesn't have multifunctions in contrast to the PDL cell that expresses the osteoblastic properties generally. However, it was reported that gingival cells contains functionally heterogeneous fibroblast subpopulations [14] and there are several investigations that focused on the gingival cells properties including the osteoblastic ones [15,16]. In their reports, gingival cells expressed the mRNA of bone morphogenic proteins (BMP)-2 and BMP-4 [16], BMP-receptor and BMP-7 [17], and showed a high alkaline phosphatase ability like PDL cells and formed mineralized tissue [18]. BMP-2, -4 and -7 are members of a family of proteins with an ability of bone formation. If gingival cells can acquire the osteoblastic properties like PDL cells, their easy availability might make it possible to regenerate PDL including cementum.

Many investigations have been carried out to develop hydroxyapatite coating onto orthopaedic and dental materials to promote rapid attachment to bone [19-20]. And some showed hydroxyapatite could be effective to enhance the properties of osteoblast. Various techniques such as plasma spray [21] have been used for coating. In this chapter, we chose alternate soaking method [22] to coat hydroxyapatite on EVA as an implant material because

of its easiness and high efficacy for modification and the responses of gingival cells on the hydroxyapatite were investigated.

## **Materials and methods**

### **Materials**

EVA film ( $\overline{M}_n = 15,000$ , 200  $\mu\text{m}$  in thickness) having 56 mol% of vinyl alcohol content was kindly supplied by Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan). These films were cut into 10 or 20 mm diameter and stored in acetone at 25 °C until usage. One w/w % type I collagen solution (pH 3.7, in 5 mM phosphoric acid) was kindly donated by Nitta Gelatin Co. Ltd. (Osaka, Japan). All other materials were of chemical reagent grade and used without further purification.

### **Sample preparation**

#### **Carboxyl groups introduction on EVA**

In order to convert hydroxyl groups of EVA into carboxyl groups, EVA films were exposed to ozone as described in chapter 2 [8]. In brief, ozone gas was produced by a generator (POX-10, Fuji Electric Co., Ltd, Tokyo, Japan) equipped with an O<sub>2</sub> cylinder and taken into a glass bottle in a water bath at 70°C at a rate of 1.0 L/min. EVA films were hung in the bottle and treated at 0.5 g/h of ozone gas flow for 5h. In this step, 0.1 $\mu\text{mol}/\text{cm}^2$  of carboxyl groups were introduced on the EVA surface (COOH-EVA), evaluated by simple neutralization titration.

#### **Collagen immobilization on the EVA**

Chapter 2 shows details on the preparation of collagen immobilized EVA (COL-EVA) surfaces [8]. EVA films having carboxyl groups were immersed in 0.5 w/w% type I collagen solution (pH 3.7, in 5mM phosphoric acid) and gently stirred at 25 °C for 16 h. After the

polyioncomplex formation, unimmobilized collagen was removed by rinsing the films with an excess amount of 5mM of phosphoric acid (pH 3.7). The amount of collagen immobilized on the EVA surface was 6  $\mu\text{g}/\text{cm}^2$ , evaluated by ninhydrin method [23].

#### **Hydroxyapatite immobilization on the EVA**

Hydroxyapatite crystals were deposited on the EVA surface according to chapter 4 [10]. In brief, EVA films having carboxyl groups were soaked in 200mM  $\text{CaCl}_2$  and 50mM Tris(tris hydroxymethyl aminomethane)-HCl solution (pH 7.4) at 25°C for 12h. Then the films were subsequently soaked in 120mM aqueous  $\text{Na}_2\text{HPO}_4$  solution (pH 9.3) at 25°C for 12h. This alternate immersion was repeated and continued for 10 times. After each cycle of the alternate immersion, the hydroxyapatite immobilized EVA (HAP-EVA) films were washed by ultrasonic wave in distilled water to remove the physisorbed hydroxyapatite. The surface of EVA films was characterized by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Spectrum One, Perkin Elmer, Inc, MA, US) in the range of 650-3050  $\text{cm}^{-1}$  and pure hydroxyapatite powder (Wako Pure Chem. Ind. Ltd. (Osaka, Japan)) was used as a control material.

#### **Calcium and phosphorus determination**

The calcium concentration on EVA films was measured by an o-cresolphthalein complexon method [24]. Briefly, hydroxyapatite immobilized EVA film ( $\phi 10\text{mm}$ ) was put into 1ml of 0.1N HCl in a test tube, followed by hydrolyzation at 120°C for 1h. Three ml of 0.88M monoethanolamine buffer (pH11.0) and 0.5ml of color developing reagent (containing 0.63 mM o-cresolphthalein complexon and 69mM of 8-hydroxyquinoline) were added to the solution and the absorbance was recorded at 570 nm.

The phosphorus concentration on EVA was evaluated by molybdenum blue method [25]. After the hydrolyzation as mentioned above, 3 ml of the reduction solution (containing

77mM ammonium ferrous sulfate) and 0.5ml of color developing reagent (containing 36mM ammonium molybdate and 102mg/ml sulfuric acid) were added to the solution. After colorization, the absorbance at 750 nm was measured.

### Cell culture

Human gingival cells were isolated from gingival tissue overlying impacted third molars that were extracted by orthodontic reasons after providing informed consent protocol approved by the institutional review board of the Institute for Frontier Medical Sciences. Gingival tissue was washed with phosphate buffer solution (PBS(-)) containing 100 U/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml gentamycin and minced into 1-3 mm<sup>2</sup> with a scalpel. The gingival tissue was placed in a tissue culture dish with 0.2 ml of Dulbecco's modified Eagle's medium (DMEM)(supplemented with 10 v/v% of fetal calf serum, 100 U/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml gentamycin), followed by putting a cover glass on the tissue. At the beginning of the culture, 2-3 droplets of DMEM were put into a side of the cover glass. Culture medium was exchanged 2-3 times every week and the culture was continued at 37°C in a 5v/v% CO<sub>2</sub> incubator. After several days, migration of gingival cells on culture dish was observed. When the cells reached confluence, they were recovered by 0.1 w/v% of trypsin containing 0.025 w/v% of EDTA in PBS(-) and subcultured. Gingival cells of 2-4th passage were utilized for this study.

To evaluate gingival cell proliferation in the different surfaces, cells were seeded onto the films of COOH-EVA, COL-EVA, and HAP-EVA, and tissue culture dishes with 20mm in diameter at the concentration of 2500 cells/cm<sup>2</sup> with 2 ml of DMEM. Cell number was evaluated by the lactate dehydrogenase (LDH) activity [26], where LDH oxidizes reduced nicotinamide adenine dinucleotide (NADH) to its oxidized form and the amount of LDH is a function of cell number. Briefly, 3 ml of cell lysate containing 2 v/v% Triton X-100 in PBS(-)



and 1 ml of reaction substrate containing 0.62 mM lithium pyruvate and 0.18 mM NADH in 50mM phosphoric acid were mixed and the decrease in the absorbance at 340 nm from 30 sec to 60sec just after mixing was recorded.

#### **Alkaline phosphatase activity**

Gingival cells were seeded onto COOH-EVA films, COL-EVA films, HAP-EVA films, and conventional tissue culture dishes with 20mm in diameter at the concentration of 8000 cells/cm<sup>2</sup> with 2 ml of DMEM. After 7 and 14 days culture, alkaline phosphatase activity at confluent state was evaluated by p-nitrophenyl phosphate method [27]. Briefly, 3 ml of cell lysate (containing 2 v/v% Triton X-100 in PBS(-)) was added to 0.5 ml of substrate solution(containing 2 mM MgCl<sub>2</sub> and 33 mM disodium p-nitrophenyl phosphate at pH9.8). After colorization for 15min at 37 °C, the reaction was stopped by adding 0.5ml of 2N NaOH and the absorbance at 405 nm was measured.

#### **Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNAs of the gingival cells cultured for 7 and 14 days were extracted using Trizol (Invitrogen Co., CA. USA) based on the method reported by Chomczynski et al [28]. Total RNA (1 µg) was incubated with 0.25 U/µl of Avian Myeloblastosis Virus (AMV) reverse transcriptase XL in the presence of 2.5 µM of random 9 mers and 1mM of dNTP mixture for 30 min at 42 °C. Obtained cDNA solution was mixed with 0.2 µM of forward and reverse primers and 25 U/ml of Taq polymerase. The PCR was employed for 35 cycles according to the following steps: (1) 30 sec at 90 °C; (2) 30 sec at 54 °C; and (3) 90 sec at 72 °C. The primers for amplification of osteocalcin(OC), osteonectin(ON), collagen type I(COL1), collagen type XII(COL12), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were shown in Table 1. The PCR products were identified and visualized after electrophoresis in 1.5 % agarose gel

Table 1 Sequences of each primer

gene		sequence 5'→ 3'
Osteocalcine(OC)	forward	AGGTGCAGCCTTTGTGTCCAAG
	reverse	GCAAGGGGAAGAGGAAAGAAGG
Osteonectine(ON)	forward	GATGAGGACAACAACCTTCTGAC
	reverse	TTAGATCACAAGATCCTTGTCGAT
Collagen type 1(COL 1)	forward	TGACGAGACCAAGAACTG
	reverse	CCATCCAAACCACTGAAACC
Collagen type 12(COL 12)	forward	TGCCTTTACCCAGACCTCAG
	reverse	CAAGTCCCTCTGAAACCCTC
GAPDH	forward	CATCACCATCTTCCAGGAGC
	reverse	CATGAGTCCTTCCACGATACC

with ethidium bromide staining. All reagents for RT-PCR were purchased from Takara Bio Inc. (Shiga, Japan).

#### **Enzyme linked immunosorbent assay (ELISA)**

After 21 days culture of gingival cells on the different surfaces, medium were exchanged to 1ml of serum free medium and further culture proceeded for 48 h. The amount of the osteocalcin, osteopontin, and osteonectin in the cells was measured by enzyme linked immunosorbent assay as follows. One hundred  $\mu$ l of cell lysate (containing 2 v/v% Triton X-100 in PBS(-)) was incubated on 96well elisa plate (IWAKI, Tokyo, Japan) for 24 h at 4 °C. After adsorption of protein, the plate was washed by PBS(-) 3 times. One hundred  $\mu$ l of 1  $\mu$ g/ml of mouse monoclonal antihuman antibodies (anti-osteocalcin (OCG4), anti-osteonectin (OSN4-2), (Takara Bio Inc. Shiga, Japan) and anti-osteopontin (IB20, IBL Co., Ltd., Gunma, Japan)) in PBS(-) was put in the plate and incubated for 2 h at 25 °C. After washing 3 times with PBS(-), 100  $\mu$ l of 1 $\mu$ g/ml peroxidase-labeled anti-mouse IgG was added and further incubation proceeded at 25 °C for 1h. Following 4 times washing, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma-Aldrich Co., MO., USA) was added and the absorbance at 405 nm was measured by a plate reader (versa max, Molecular Devices Co., CA., USA).

## **Results and discussion**

### **Hydroxyapatite immobilization onto EVA**

Hydroxyapatite crystals were deposited on the COOH-EVA surface by following mechanism detailed in chapter 4 [10]. Firstly, calcium ions were adsorbed on the carboxyl groups of EVA. The adsorption of calcium ions dominated that of phosphorus by the chemical equilibrium such as chelate formation between  $\text{Ca}^{2+}$  and  $\text{COO}^-$  because no

adsorption of calcium was observed, when EVA without carboxyl groups was used. The amount of the calcium and the phosphorus adsorbed on the EVA by the alternate soaking was given in the Fig.1 (A), (B), respectively. Both the amounts of calcium and phosphorus increased with an increase in the alternate soaking times as well as the amount of the carboxyl groups on EVA. Beyond 4 times soaking, a thin layer of calcium deposition was clearly observed on the EVA surface. Hydroxyapatite crystal formation was multiplied during this cycle. After 10 times, a lot of small crystals on the film become completely white. As is mentioned above, no carboxyl groups made any hydroxyapatite deposition. Table 2 shows the molar ratio of the calcium to phosphorus and the value was around 1.6, regardless of cycle number, though, which was lower than that of pure hydroxyapatite (1.67). So the immobilized calcium phosphate consisted of various species such as hydroxyapatite, tricalcium phosphate or carbonate apatite. In fact, a small peak of ATR-FTIR spectrum assigned to carbonate (asterisk) was observed for HAP-EVA in Fig. 2, which was characterized by Dorozhkina et al [29]. In the alternate soaking method, it was thought that the crystals formation was considerably rapid and the small and various types of calcium phosphate were formed.

### **Cell proliferation**

Proliferation of gingival cells on various substrates was given in Fig.3. Gingival cells showed the highest proliferation on the tissue culture dish. In contrast, on the HAP-EVA cell growth was slightly inhibited compared with other surfaces. Chapter 3 described the biocompatibility of EVA was markedly enhanced by collagen immobilization [9]. Collagen is known to be biocompatible material and used to the scaffold of the cells and growth factors on the tissue engineering. However, no growth enhancement effect of collagen was observed in gingival cells. HAP shows no cytotoxicity and is well applied in the

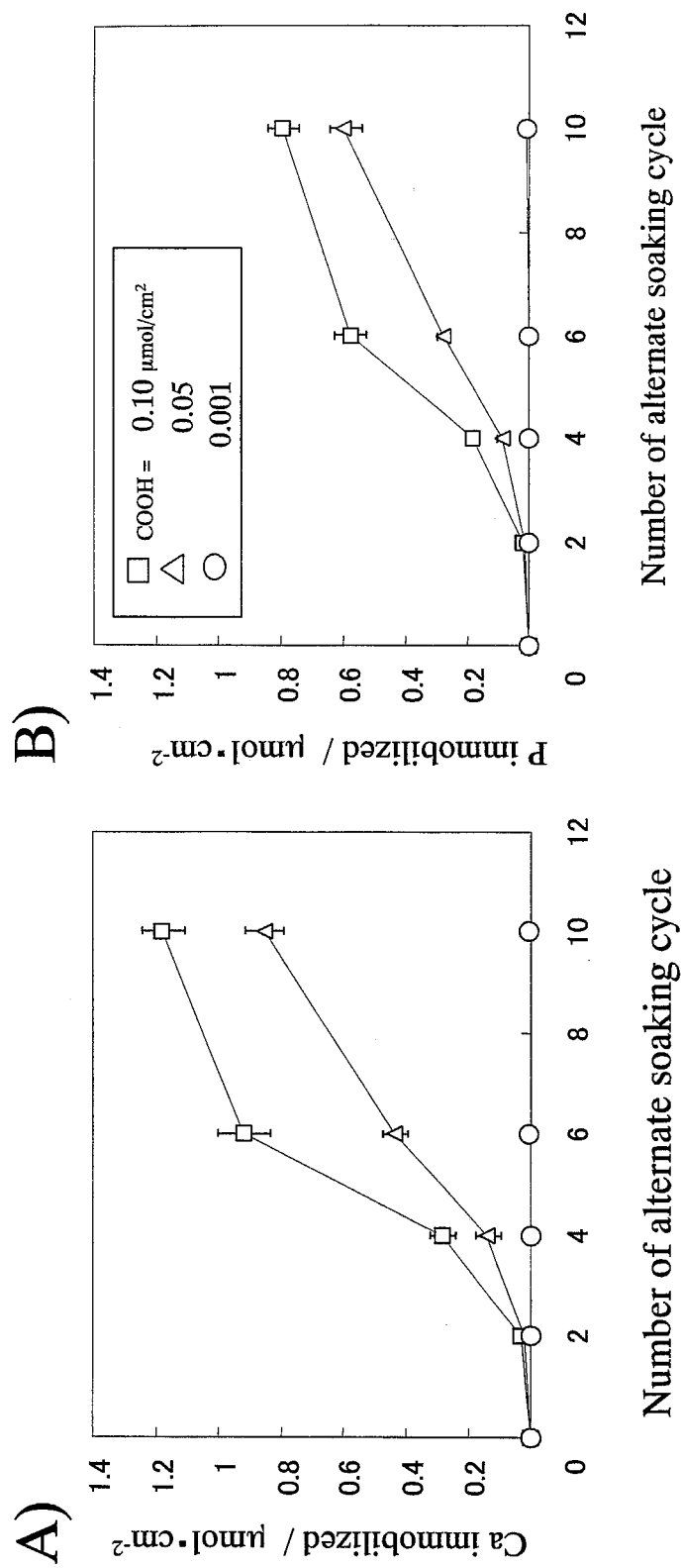


Fig. 1 Relationship between alternate soaking cycle and (A) calcium and (B) phosphorous immobilization on COOH-EVA with various amount of carboxyl groups. The error bars indicate the SD (n=3).

Table 2

The molar ratio of calcium to phosphorous in the crystallites immobilized on COOH-EVA

Cycle number / time	2	4	6	10
Ca/P	1.60	1.60	1.51	1.62

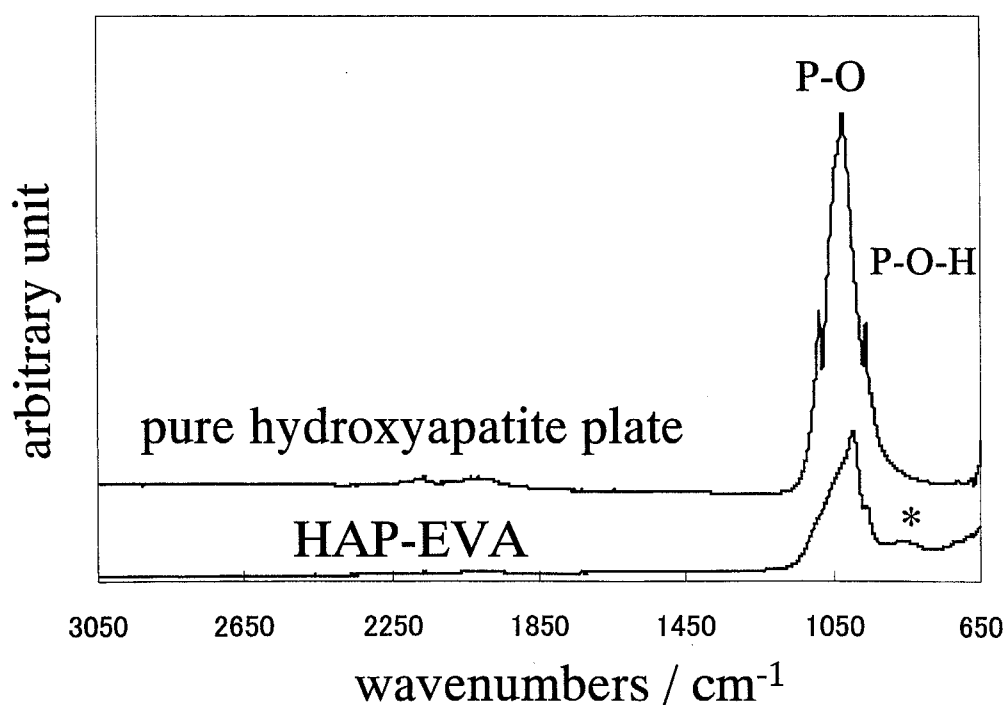


Fig. 2 ATR-FTIR spectra of the surface of HAP-EVA and pure hydroxyapatite. The band marked by asterisk around 850  $\text{cm}^{-1}$  could be assigned to carbonate.

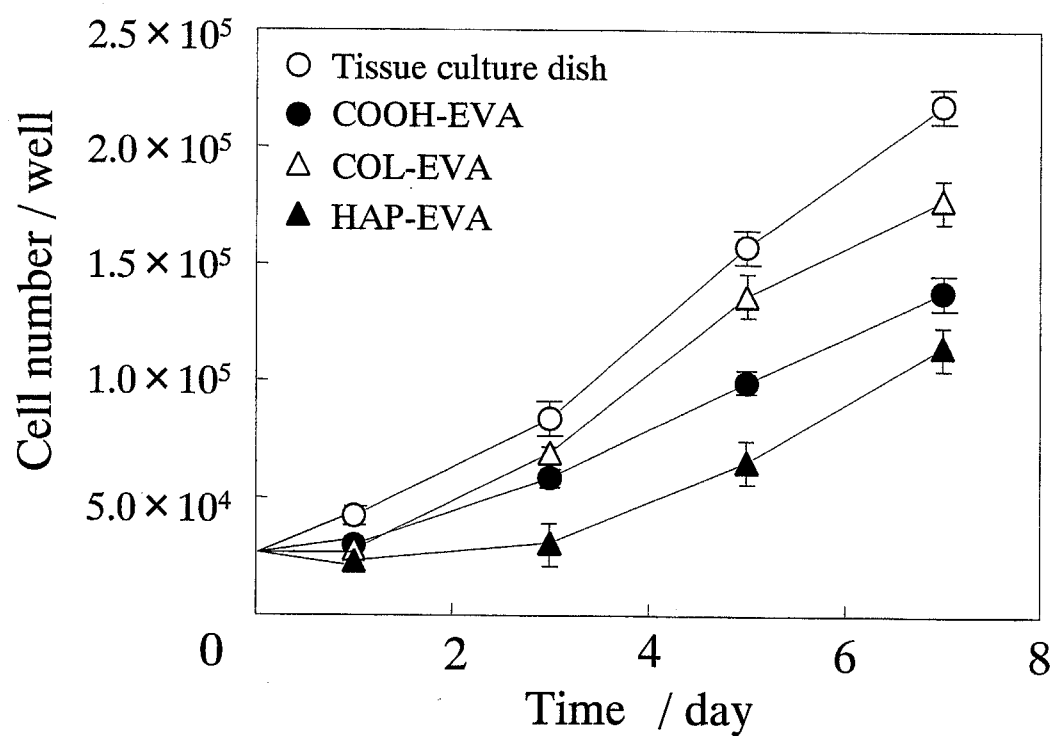


Fig. 3 Gingival cell growth on the various surfaces. The error bars indicate the SD ( $n=3$ ).

orthopaedic and dental implants for direct connection and fixation between bone and implant surface [20]. In Chapter 4, the author has showed the PDL cells were differentiated toward osteoblast-like cells by culturing on the HAP-EVA though their growth was suppressed [10]. Therefore, it is likely that low growth rate of gingival cells on the HAP-EVA suggests the cell differentiation on HAP.

#### RT-PCR

The results of RT-PCR of gingival cells cultured on various surfaces were shown in Fig 4. The mRNA of osteocalcin was expressed in the culture only on the HAP-EVA for 2 weeks. Osteocalcin is the most abundant noncollagenous protein localized in mineralized tissue and plays an important role in bone remodeling. This calcium binding protein represents the peculiar biochemical marker of final differentiation of osteoblast and is produced by also odontoblast and cementoblast. A number of reports show that osteoblasts are influenced by HAP [20,30]. In contrast, osteonectin, collagen type I and XII were expressed, regardless of the surfaces. Osteonectin is a noncollagenous protein of bone matrix that is quite ubiquitously expressed as is in odontoblasts. It is reported that osteonectin is expressed during earlier stages of differentiation than osteocalcin [31]. These results indicated that gingival cells having osteoblastic and osteonectin positive property could be potent of osteocalcin mRNA expression when cultured on the HAP-EVA. Collagen type I is well-known protein that widely distributed in the soft and hard tissue. And expression of collagen type XII is reported in dense connective tissues like PDL predominantly composed of collagen type I. Collagen type XII expression is activated after tooth eruption and limited to the mature PDL matrix. This protein is essential to PDL because its mutation induces disruption of PDL matrix [32,33]. Expression of these two types of collagen in gingival cells suggests the potential to maintain and regenerate the PDL.



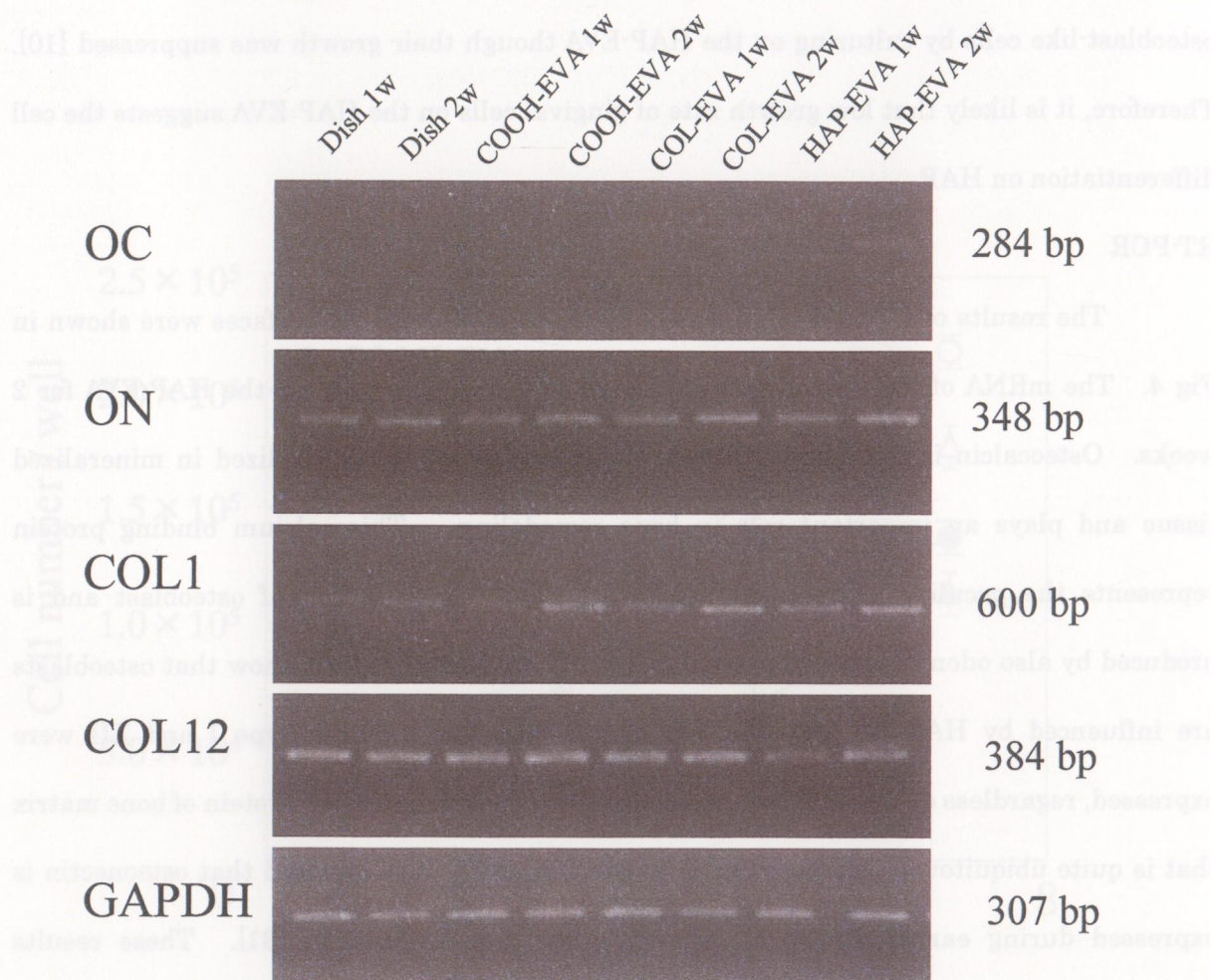


Fig.4 RT-PCR analysis of gene expression of gingival cells cultured on various surfaces. bp; base pairs.

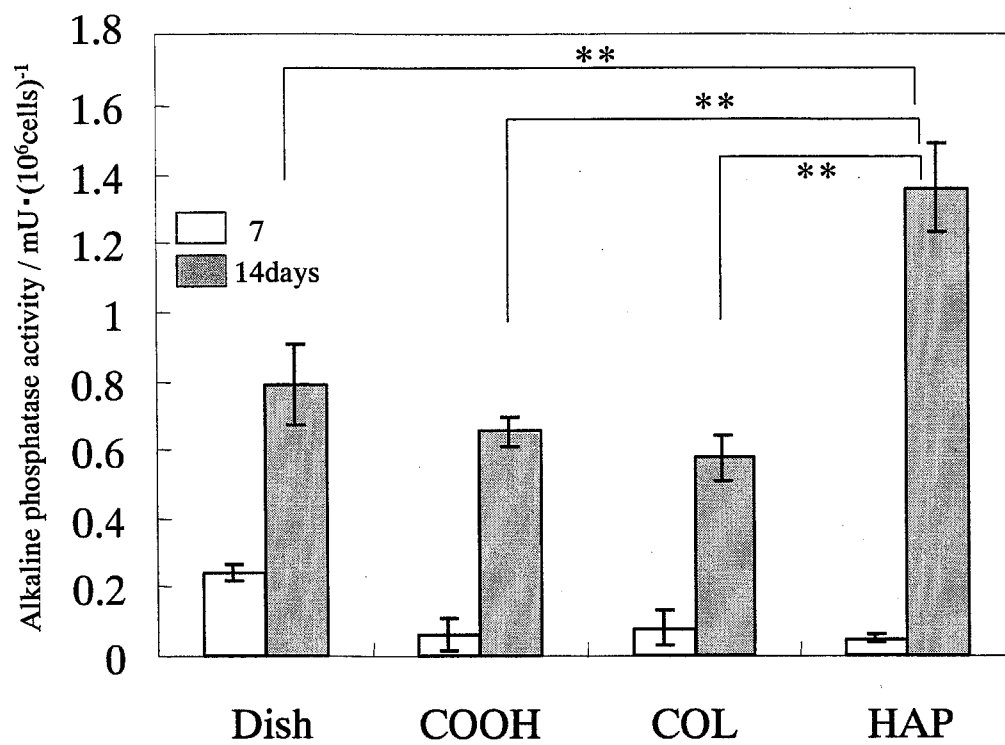


Fig.5 Alkaline phosphatase activity of gingival cells on the various surfaces. The error bars indicate the SD (n=3). \* \* p<0.05

Furthermore, culturing on HAP-EVA could make gingival cell possess the osteoblastic or cementoblastic properties.

#### **Alkaline phosphatase activity**

Alkaline phosphatase activity of gingival cells cultured on the different surfaces at 7 and 14 days is given in Fig. 5, where data were normalized by the cell number. Alkaline phosphatase is a glycoprotein, mineralizing in the calcified tissue such as bone and cementum [34]. At 7 days culture, no significant difference in alkaline phosphatase activity was observed. In contrast, its activity was the highest on the HAP-EVA and the lowest on the COL-EVA at 14 days. In this experiment, cells were seeded on the materials to be confluent in 7 days and no proliferation occurred from 7 to 14 days, which might suggest the differentiation of the cells during this period.

#### **ELISA**

In order to evaluate the osteoblast properties in gingival cells, osteocalcin, osteopontin, and osteonectin secretion were estimated for gingival cells cultured on the different surfaces for 21 days and the results were given in Fig 6-8. In this evaluation, the concentrations of each protein cannot be calculated and only absorbance per cell was given, because of the difficulty in obtaining the pure antigen. Comparison of culture surfaces is easily available from the absorbance at 405 nm. HAP-EVA showed significant intensity in osteocalcin, osteopontin and osteonectin secretion compared with COL-EVA, COOH-EVA, and tissue culture dish. Osteopontin is an also major protein in bone and has a cell adherent property due to Arg-Gly-Asp (RGD) sequences, which is reported to be contained in several tissue such as kidney, nerve, and mammary gland in addition to mineralized tissue [35]. In osteogenesis, osteopontin is expressed during matrix formation. Since osteopontin is expressed in alveolar bone and cementum, its differential expression indicates the

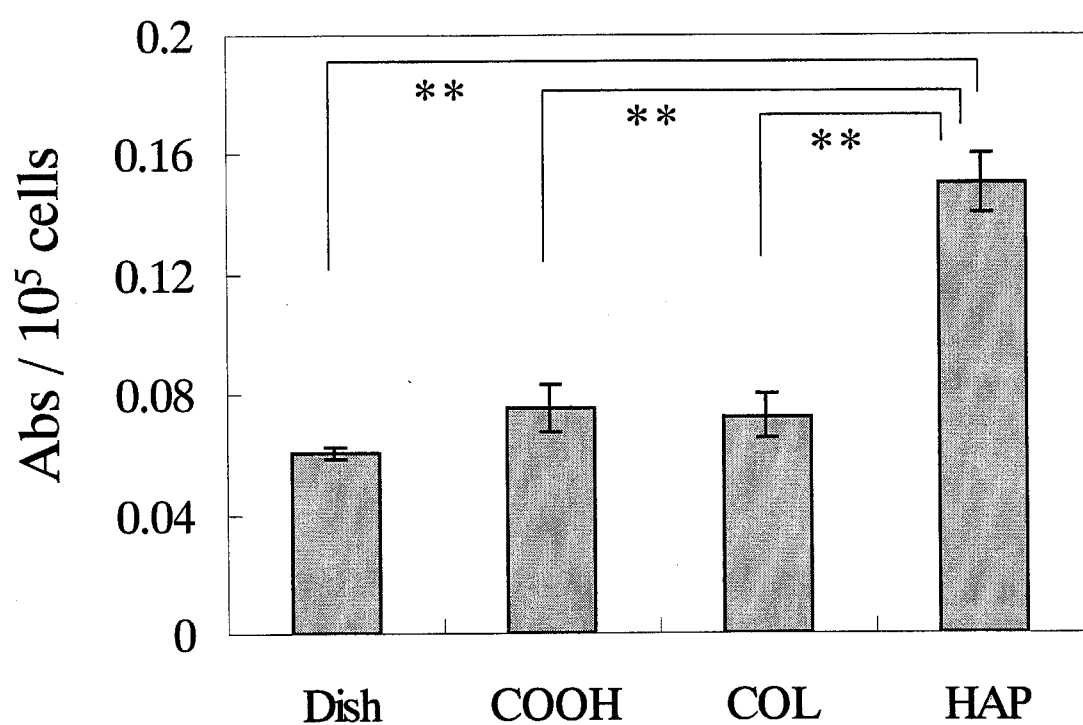


Fig.6 Osteocalcin secretion of gingival cells on the various surfaces. The error bars indicate the SD (n=8). \* \* p<0.05

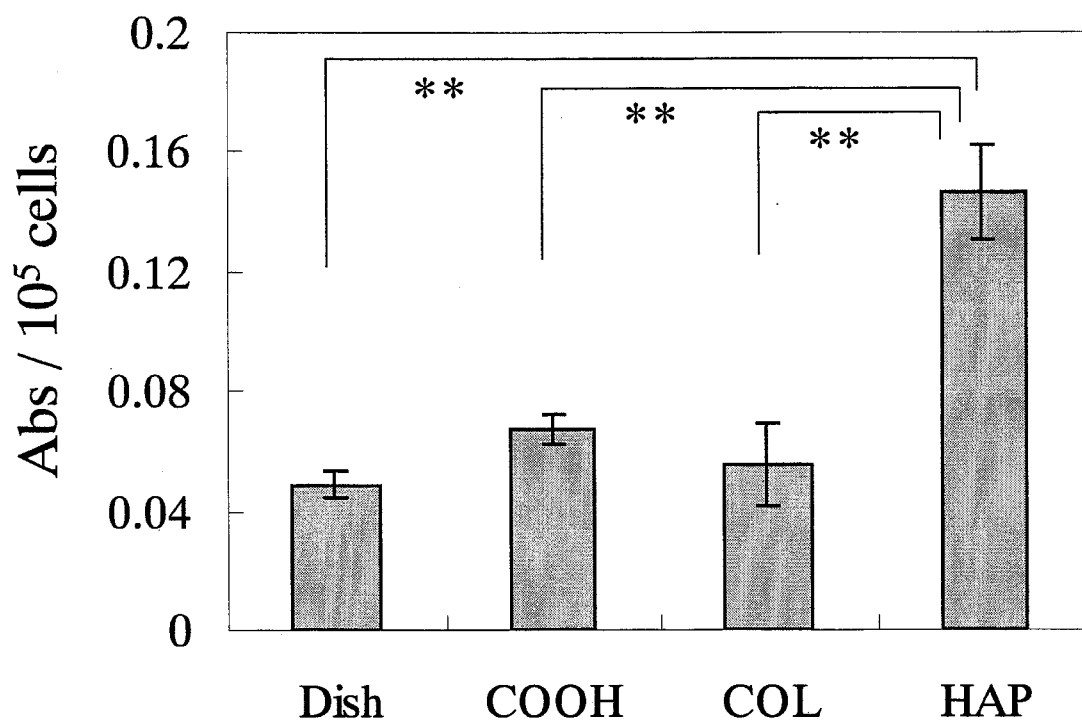


Fig.7 Osteopontin secretion of gingival cells on the various surfaces. The error bars indicate the SD (n=8). \* \* p<0.05

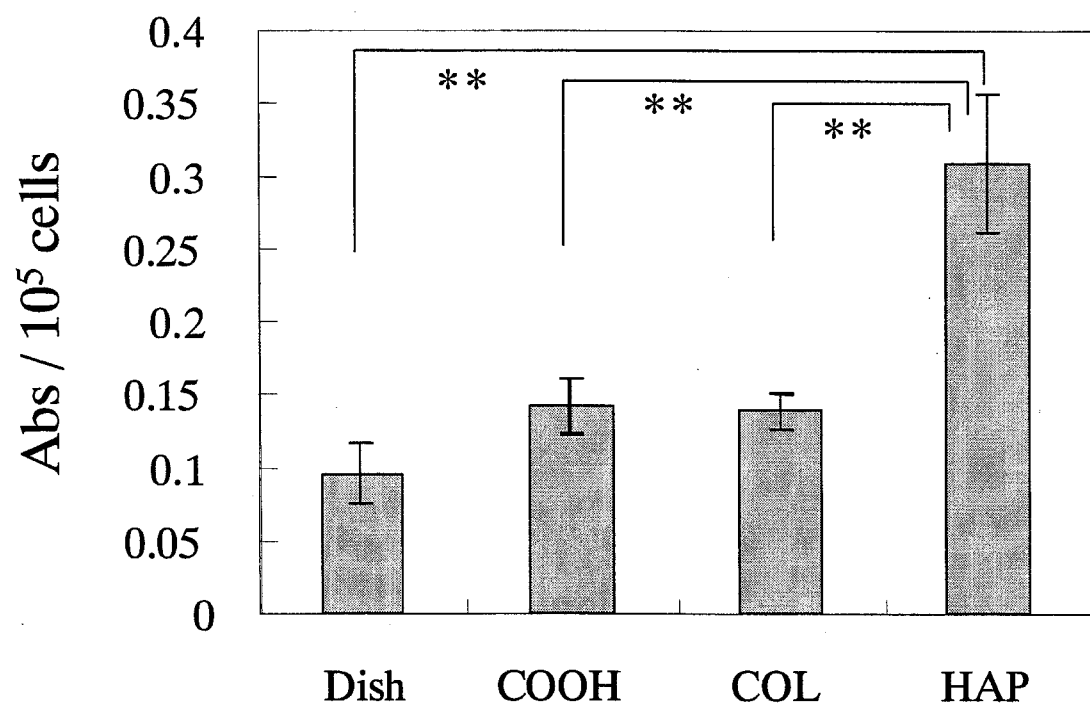


Fig.8 Osteonectin secretion of gingival cells on the various surfaces. The error bars indicate the SD (n=8). \* \* p<0.05

acquisition of osteoblast-like cell properties and formation and repair of periodontal tissues [36]. As mentioned above, osteocalcin and osteonectin are also important protein to maintain and regenerate periodontium including bone and cementum [37]. It was reported that the titania / hydroxyapatite composit made mature osteoblast-like cell MG63 enhance the osteocalcin secretion ability. And chapter 4 suggested that PDL cells were differentiated to bone-like cells cultured on the HAP-EVA. The result of alkaline phosphatase activity suggests that gingival cells have an ability to differentiate into osteoblast-like cells when cultured on HAP-EVA.

## Conclusion

Hydroxyapatite crystalline could be immobilized on EVA films through ozone treatment and alternate immerse method. Although the gingival cell growth on the HAP-EVA was lower than those on COL-EVA, COOH-EVA, and tissue culture dish, the mRNA of osteocalcin in gingival cells was expressed only by culturing on HAP-EVA. The mRNA of osteonectin and collagen type I and XII were expressed on all of the surfaces. Increment in peculiar osteoblast markers such as alkaline phosphatase and osteocalcin, osteopontin and osteonectin expression suggest that HAP-EVA might stimulate gingival cells to differentiate into to osteoblast-like cells properties. Therefore, it was summarized that the gingival cell might have maintain and regenerate the periodontium including of cementum and alveolar bone such as PDL cells on HAP-EVA, which might make it possible to prepare further highly organized hybrid implant possessing PDL and cementum on the surface of artificial dental implant.

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## **Chapter 6**

### **Periodontal Ligament Regeneration around Hybrid Implant; Containing Organic Material and PDL Cells**

#### **Introduction**

Implantation of an artificial tooth root has been widely performed in dental surgery to recover the lost functions of a natural tooth. Currently, osseointegrated type implant is acceptable because it can be tightly fixed with an alveolar bone [1]. However, the direct impact of occlusal and bruxing forces has often caused absorption of the alveolar bone and loosening of the implant [2]. In contrast, the root of a natural tooth is covered with a periodontal ligament (PDL), mainly consisting of active fibroblasts and collagen fibers [3,4], and plays quite an important role as a shock absorber [5] against the mastication impact and as a receptor of forces. If an implant possessing the periodontal ligament could be developed, these all problems would be resolved and the dental treatment might be changed basically. Buser et al. [6] reported that collagen fibers inserted into the cementum-like tissue on the implant surface when the implant was placed in contact with adjacent tooth periodontium, and the result suggested that the retained tooth root could serve as a source for cells, participating in the healing process. However, it is still unknown how is the interface between the collagen fiber and implant surface that was bioinert inorganic material. Nyman et al. [7] demonstrated that PDL cells have the capacity to form periodontium. This study was aimed at developing the newly hybrid implant using organic material possessing the PDL around itself by tissue engineering technique. The concept is schematically summarized in

Fig. 1. First, a titanium implant is coated with poly(ethylene-co-vinyl alcohol)(EVA) [8] and surface modification is employed to enhance the biocompatibility of EVA through collagen immobilization [9] and collagen sponge coating. Next, the PDL cells are cultured on the modified implant [10] and the specimen is implanted into a jawbone. PDL regeneration is expected to take place around the artificial tooth root covered with organic materials. The adhesive strength between EVA and titanium was very high (about 15MPa) because of its hydrophilicity due to hydroxyl groups [8] and the hydroxyl group is easy to convert into other functional groups such as carboxyl [11,12] and amino groups [13]. It is well known that biocompatibility can be improved by collagen immobilization onto surfaces having those groups [14]. Therefore, it is expected that the collagen fiber of regenerated periodontal ligament will connect immobilized collagen on the hybrid implant and PDL cell culture on collagen-immobilized EVA makes it easy to regenerate periodontal ligaments.

## Materials and methods

### Materials

In this chapter, pure titanium implants (cylindrical shape implant with 3.0 mm in diameter and 9.5 mm length with mirrored surface) were purchased from Platon Japan Co. (Tokyo, Japan). EVA ( $M_n = 15,000$ ) having 56 mol% of vinyl alcohol content was kindly supplied by Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan). One w/w % type I collagen solution (pH 3.7, in 5 mM phosphoric acid) was kindly donated by Nitta Gelatin Co. Ltd. (Osaka, Japan). All other materials were of chemical reagent grade and used without further purification.

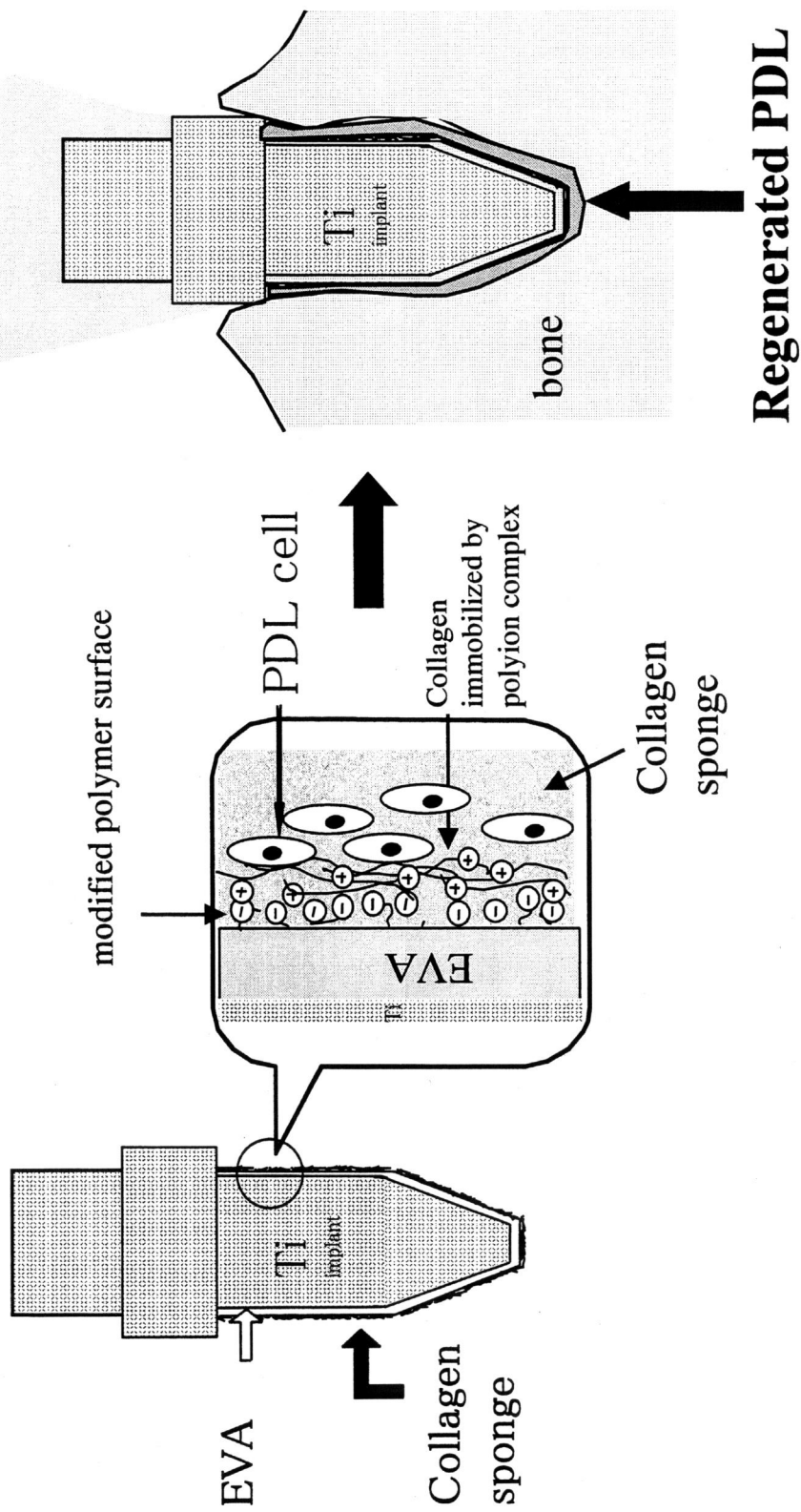


Fig.1 . A schematic illustration of the regeneration of periodontal ligament

## Cell culture

The mandibular first, second, and third premolars of a canine were extracted and stored in phosphate buffer solution (PBS(-)) containing 100 U/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml gentamycin at 4 °C immediately after extraction. The periodontal ligament attached to the mid-third of the root was minced (1-3 mm<sup>2</sup>) and recovered with a scalpel to avoid contamination by gingival and nerves cells. PDL tissue was placed in a 35 mmφ tissue culture dish with 0.2 ml of Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10 v/v% of fetal calf serum, 100 U/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml gentamycin), followed by putting a cover glass on the tissue. At the beginning of the culture, 2-3 droplets of DMEM were put into a side of the cover glass. Culture medium was exchanged 2-3 times every week. The cultures were kept at 37°C in a incubator of 5v/v% CO<sub>2</sub>. After several days, migration of PDL cells on culture dish was observed. When the cells reached confluence, they were recovered by 0.1 w/v% of trypsin and 0.025 w/v% of EDTA in PBS(-) and subcultured. PDL cells of 2-3rd passage were utilized in this study.

## Sample preparation

Titanium implants were treated by 30 w/w % aqueous H<sub>2</sub>O<sub>2</sub> solution at 60 °C for 24 h to enhance the adhesive strength between EVA and titanium. Then the surface modified implant was dipped in 10w/w% EVA solution (in n-propanol/H<sub>2</sub>O = 7/3, w/w) and dried in air. As EVA has hydroxyl groups on its surface, the carboxyl groups could be easily introduced on the surface by oxidation. Author selected the ozone gas exposure because uniform oxidation was available even in a cylindrical shape. Chapter 2 shows the 0.1 mmol/cm<sup>2</sup> of carboxyl groups were introduced by ozone oxidation at 64 °C for 6 h [8]. And then, collagen was

immobilized through polyioncomplex onto the carboxyl groups introduced EVA coated implant and collagen sponge was coated following freeze-drying process. Collagen sponge coated implants were thermally treated at 160 °C for 8 h in vacuum to crosslink and sterilization. The photographs of these implants were shown in Fig.2. (A) and (B) shows an intact and collagen sponge coated implant respectively. The scanning electron microphotograph of the collagen sponge is given in Fig.2 (C).  $10^5$  of PDL cells obtained from each canine were seeded on to the collagen sponge coated implants and implanted in to the mandibular jaw of each canine after 2 week culture. Cell number on the implant was evaluated by the lactate dehydrogenase (LDH) activity [15], where LDH oxidizes reduced nicotinamide adenine dinucleotide (NADH) to its oxidized form and the amount of LDH is a function of cell number. Briefly, each implant after culture of cells was put into 3 ml of 2 v/v% Triton X-100 /PBS(-) and 1 ml of substrate containing 0.62 mM lithium pyruvate and 0.18 mM NADH in 50mM phosphoric acid was mixed, and the decrease in the absorbance at 340 nm from 30 sec to 60sec just after mixing was recorded. In addition, to make a histological specimen more easily, EVA itself was mold to cylindrical shape like a titanium implant and then the ozone treatment, collagen immobilization, collagen sponge coating, and PDL cells culture was carried out as is mentioned above.

### Animal experiments

Three adult hybrid canines of 10-14 kg weight were used. This experiment was performed in accordance with the Guidelines for Animal Experiments of Kyoto University (1989). The canines were anesthetized with an intramuscular injection of 5mL of 1% ketamine and 2% of xylazine mixture. The mandibular first, second, and third premolars of each canine were extracted bilaterally. Approximately 2 months after teeth extraction, PDL cells seeded implants were implanted in the edentulous regions. Three implants were



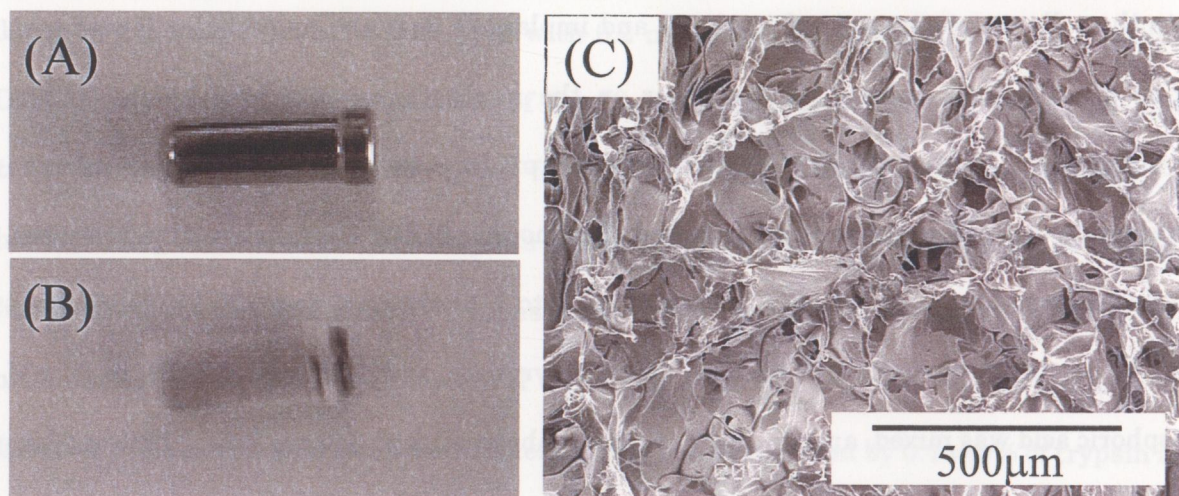


Fig.2 Photographs of (A) intact, (B) collagen sponge coated implant, and (C) scanning electron microphotograph of the surface of collagen sponge.



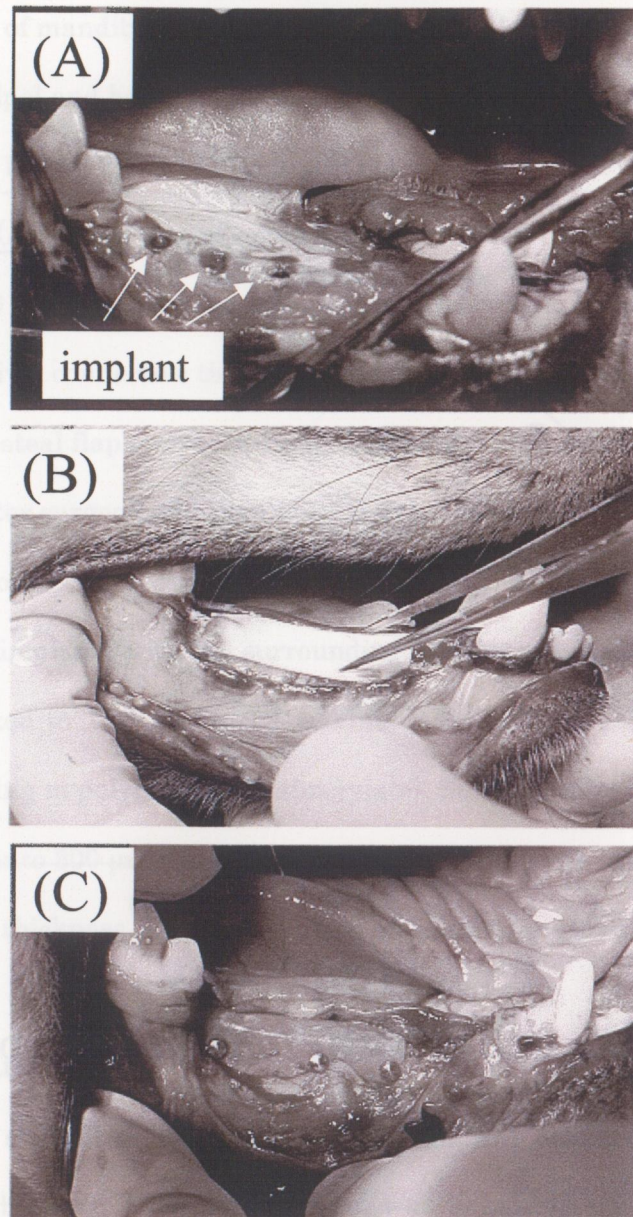


Fig.3 Photographs of implantation of test specimens into a canine. Hybrid implants were (A) placed in the jaw bone, (B) covered with membrane, and (C) with membrane fixed with pins.

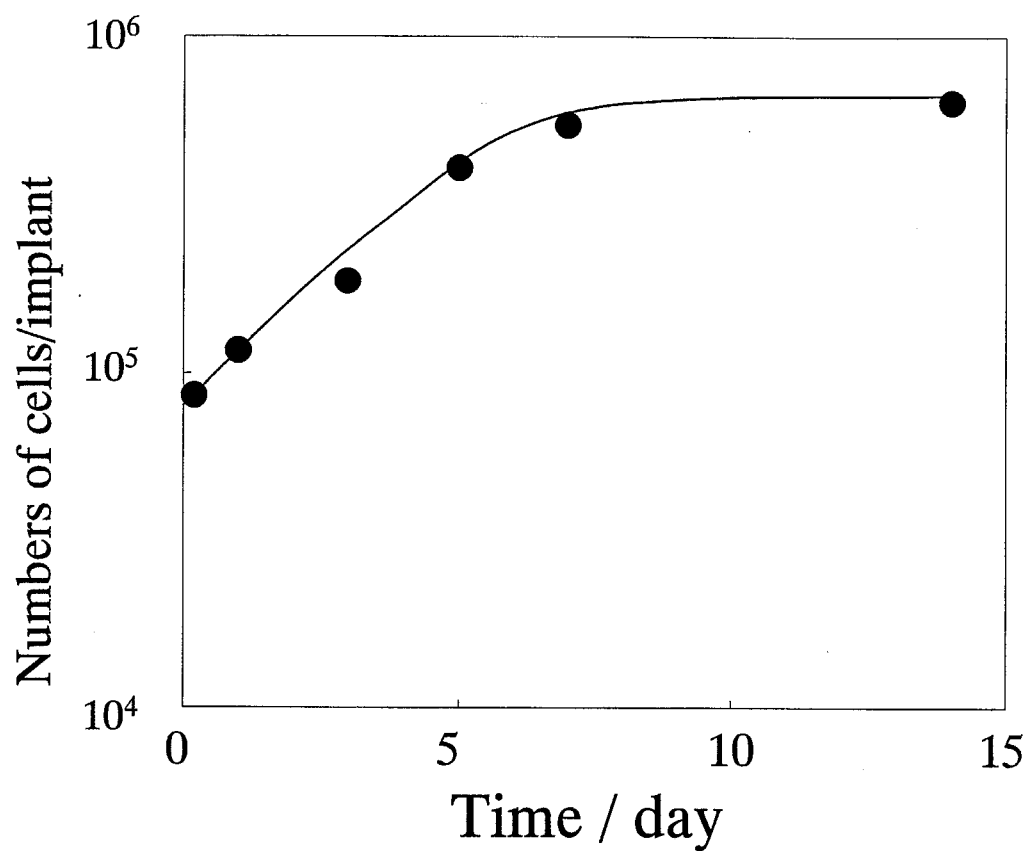


Fig.4 PDL cell growth in collagen sponge around the implant.

applied to each side of mandibular of canine. The edentulous region was opened by a crestal incision using a scalpel and bored a hole using a low speed drill (3.25 mm $\phi$ ). The hole width was about 100 $\mu$ m larger than the implant not to peel off the collagen sponge during implantation (Fig.3(A)). The placed implants were covered by a biodegradable guided bone regeneration (GBR) membrane (GC membrane®, GC Corp., Tokyo, Japan) to prevent a downgrowth of gingival connective tissue into the wound around the implant (Fig.3(B, C)) and then, the mucoperiosteal flaps were sutured. The canines were fed a pellet diet swollen with milk for 1 month after surgeon and then normal dried pellet diet.

After 3 months, the canine was sacrificed by KCl intravenous injection under anesthesia and the implants with the surrounding bone were recovered. The specimens were rinsed in saline, fixed in 10% buffered formalin, dehydrated in ethyl alcohol, degreased in acetone and embedding in poly(methyl methacrylate). Sections were cut in the midaxially in a buccolingual plane into 300  $\mu$ m using a diamond band saw. The specimens were ground and polished to have a thickness of approximately 50  $\mu$ m and stained with sirius red and fast green [16].

## Results and discussion

Chapter 2 showed that the titanium surface was oxidized to Ti-OH by H<sub>2</sub>O<sub>2</sub> and adhesion strength between EVA and titanium was enhanced up to 15 MPa due to their hydrophilicity [8]. Through following immobilization of collagen, the titanium implant has an organic surface with highly biocompatibility. There were some investigations that concluded the periodontium was regenerated around implant. Choi [17] and Urabe [18] reported that connective tissue inserted into regenerated cementum was perpendicularly arranged with titanium implant. Buser and Warrer [19] demonstrated that a cementum layer inserting collagen fiber was formed around the titanium implant, contacted with

neighbor retained tooth root. However, they did not mention about the interface between titanium and regenerated periodontium. As titanium is an inorganic material and is likely to give low bioaffinity with an organic tissue such as collagen fiber or cementum, author proposed a hybrid implant consisting of an inorganic implant covered with organic material and cells seeded on its surface to make a regeneration of periodontium easy.

PDL cell growth on the hybrid implant was given in Fig.4. PDL cells proliferated well in the collagen sponge for 2-week culture. The collagen sponge coated around implant had about 200  $\mu\text{m}$  in thickness and about 100  $\mu\text{m}$  pore size (Fig.2(C)). After 1-week culture, cell number reached  $10^6$  and proliferation almost stopped. PDL of a canine natural tooth was given in Fig.5. As is seen, periodontal ligament connected alveolar bone and tooth by Sharpey's fiber. One end of the fiber is inserted into bone and the other end is into cementum of the root surface. Fig.6 shows the histological finding of 3 months after implantation without GBR membrane and new connective tissue formation was seen between titanium body and alveolar bone. However, the direction of the fiber was parallel with implant over alveolar crest. Gingival cells growth rate is relatively faster rather than that of PDL cells, which might be from the epithelium. Gingival cells from epithelium invaded the gap between implant and surrounding bone and would take the place of PDL cells pre-cultured in collagen sponge. Therefore, GBR membrane was used for prevent the fibrous tissue invasion by covering the gap. Fig. 7 shows the photograph of the surface of intact EVA implant as a control. Clearly, it shows the parallel fiber with implant. That is not by downgrowth of gingival tissue but by fibrous encapsulation due to the exclusion. The microphotograph around hybrid implant after 3 months was given in Fig.8. It was seen that the collagen fiber was arranged perpendicularly from the boundary of implant to alveolar bone (A). (B) is a photograph of another implant in a different canine and shows clearly that collagen fiber



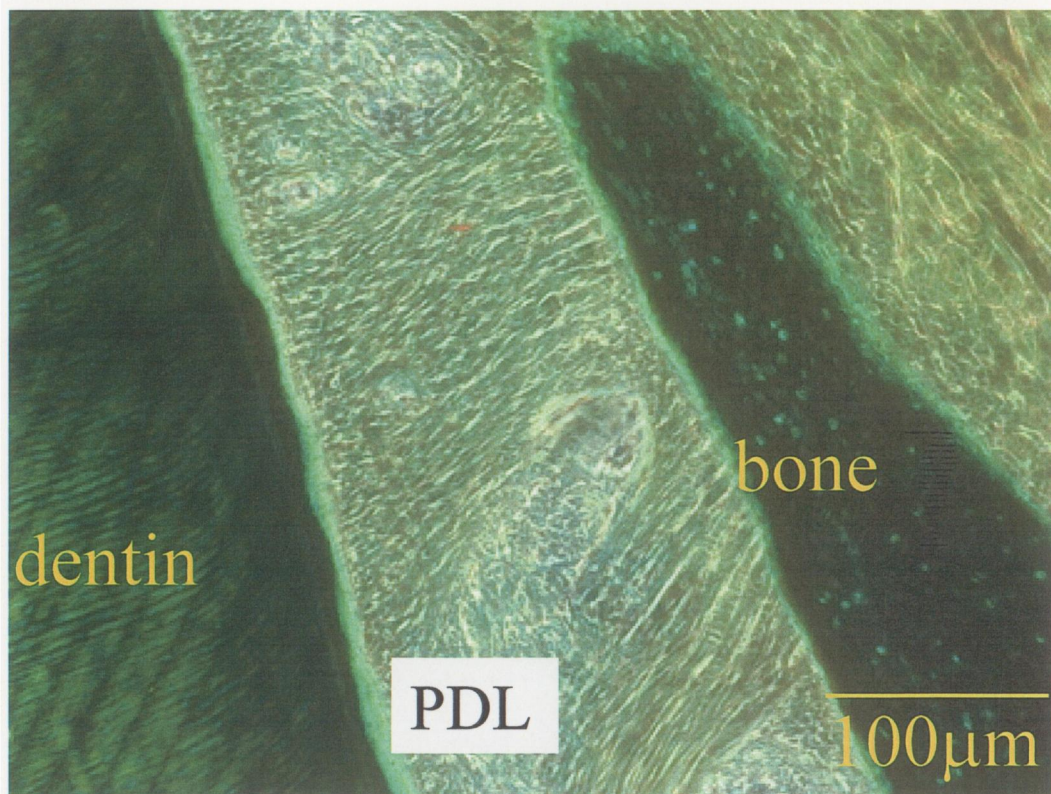


Fig.5 Histological micrograph of a canine natural tooth root.



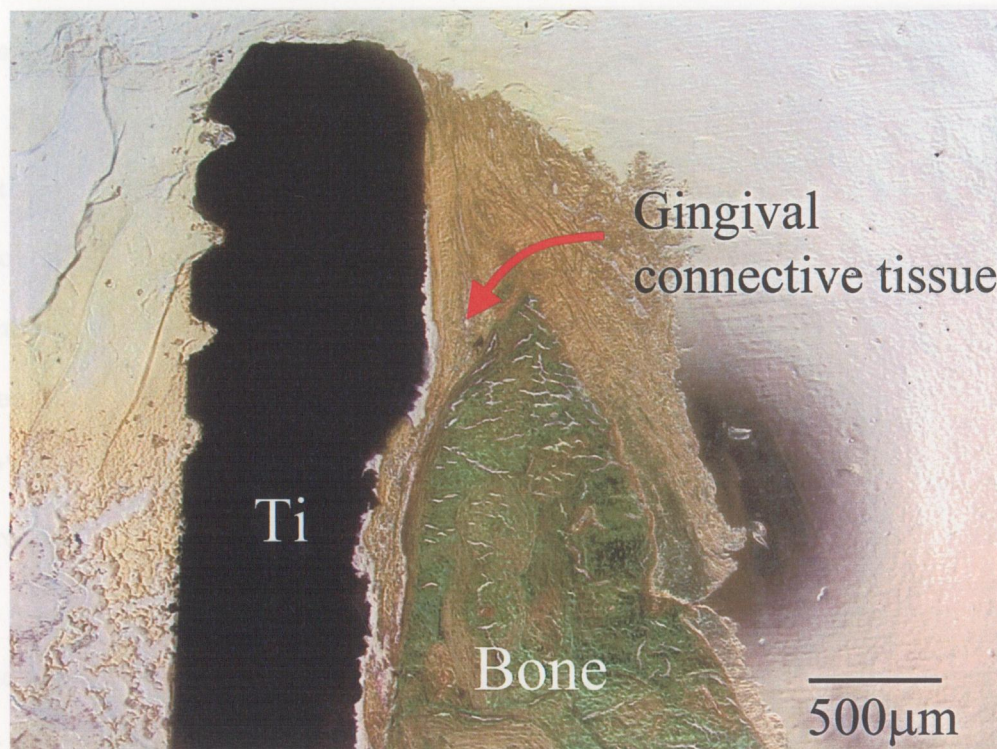


Fig.6 Histological micrograph of hybrid implant without GBR membrane 3 months after placement.



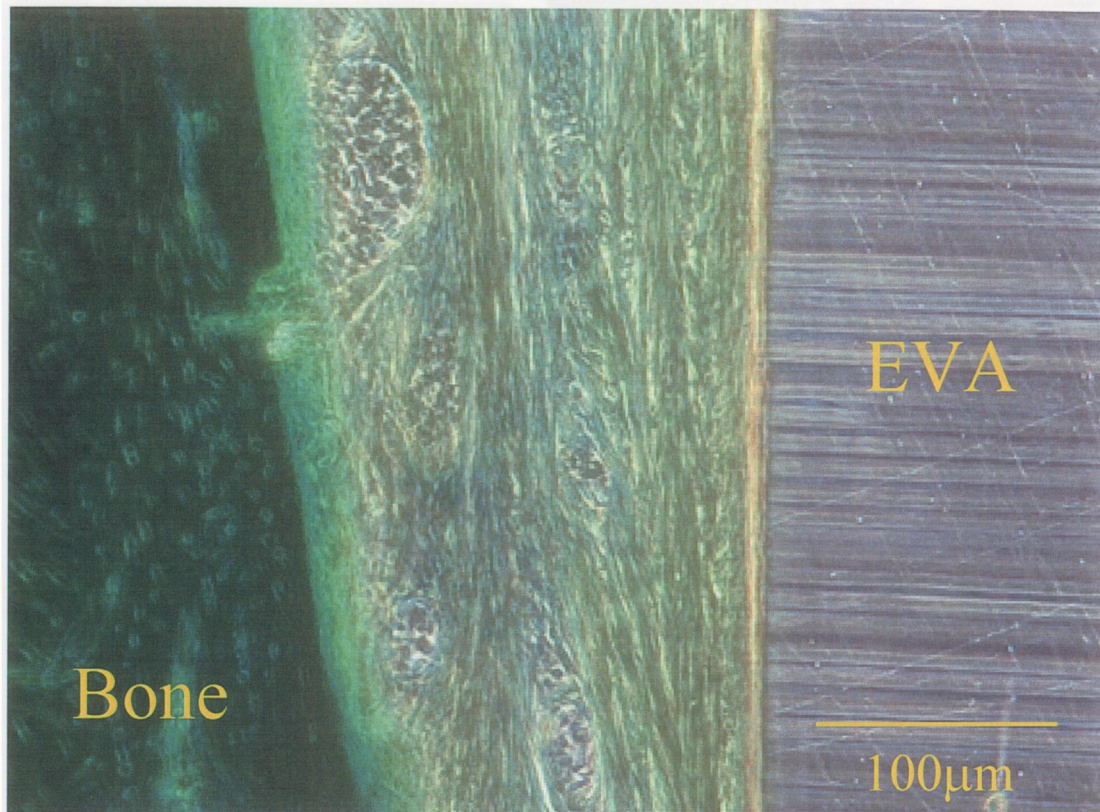


Fig.7 Histological micrograph of EVA implant without culture of PDL cells 3 months after placement with GBR membrane.



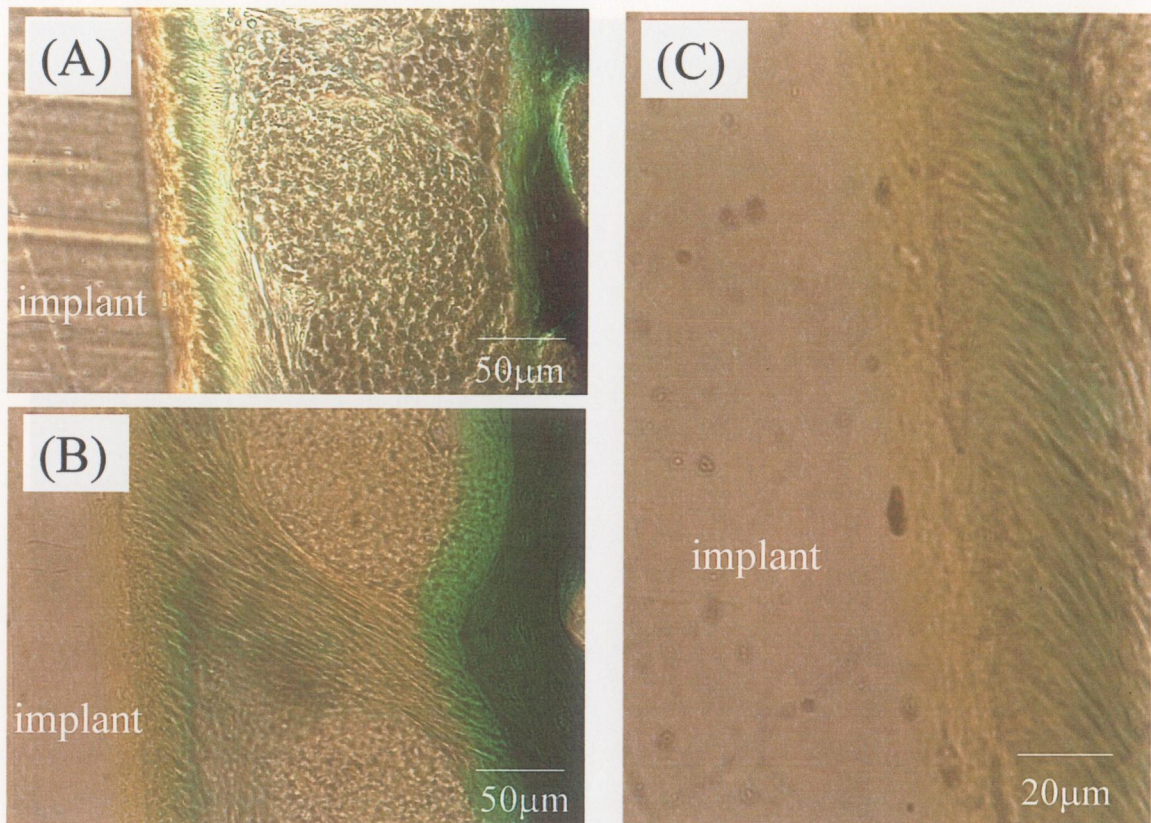


Fig.8 Histological micrograph of hybrid implant with cultured PDL cells 3 months after placement. (A) perpendicularly arranged collagen fiber (B) connection between alveolar bone and implant surface with collagen fiber (C) a higher magnification of the implant surface, where collagen fiber was continuously connected with EVA surface.

connected implant surface and bone such like PDL of natural tooth. Many studies about regeneration of PDL have demonstrated. Buser et al. showed formation of fibrous tissue around titanium implant in the presence of adjoining root tips whose periodontium was remained [19]. Urabe et al. reported the morphogenetic behaviour of periodontium around hydroxyapatite implant [18]. In the present study, it was interesting that the interface of EVA and regenerated PDL was not so clear (C), different from above reports, suggesting that highly biocompatible EVA and PDL cells could have attached closely. Generally, PDL fiber was buried into cementum tissue. In this investigation, there are some tissues like cementum around the interface between regenerate PDL and EVA, though they could not be confirmed as cementum tissue through the photographs yet. EVA has high affinity to hydroxyapatite, which is main component of cementum, and PDL cell have an ability to generate whole periodontium, so it will be possible to regenerate periodontium including the cementum around the hybrid implant. Fig.9 shows the degree of perpendicularly arranged collagen fiber around the implant calculated according to the following formula, (length of perpendicularly arranged collagen fiber / circumference length of the root part of implant) × 100. Comparing with the intact implant that showed no regeneration of PDL, the hybrid implant has almost 30% regenerated PDL. It strongly suggested that the hybrid implant had possibility of regeneration of periodontium around itself.

## Conclusion

Collagen fibers around the hybrid implant arranged vertically to the alveolar bone similar to those around a natural tooth. Regenerated PDL were observed around the root part of hybrid implant by ca. 30%, indicating that the hybrid implant containing organic material and PDL cells shows the possibility of regeneration of PDL around itself.

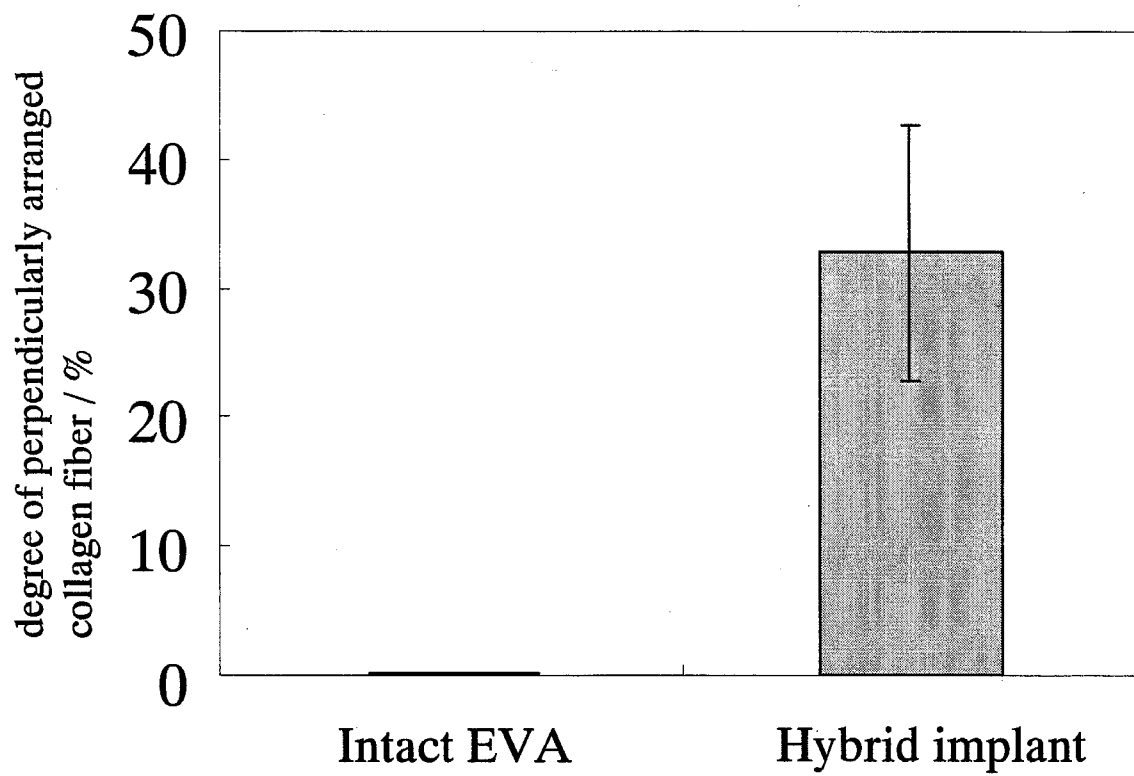


Fig.9 Degree of perpendicularly arranged collagen fibers around the hybrid implant.

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## Summary

### Chapter 1

The purpose of this study is to regenerate the human periodontal ligament (PDL) around dental implants using a hybrid structure of cells with polymer scaffold covered with a titanium implant body. In this chapter, lap shear adhesive strength between a titanium and various kinds of commercial polymers was evaluated to reveal the mechanism of the adhesion and to select a covering material having a high affinity. Among them, poly(ethylene-*co*-vinyl alcohol)(EVA) showed the highest strength. The results of electron spectroscopy for chemical analysis (ESCA) and the contact angle to water indicated that the high adhesive strength of EVA might be due to its high hydrophilicity. Water resistance of the adhesion by water immersing at 37 °C was investigated. In the case of polyurethane-titanium, the adhesive strength decreased immediately. In contrast, EVA-titanium kept its initial adhesive strength for at least up to 1 month. It was confirmed that surface modification of titanium by hydrogen peroxide enhanced the adhesive and peeling strength. It was based on not only an increase in surface adhesive area, but also an increase in the hydrophilicity of titanium by the production of Ti-OH. It was concluded that EVA was selected to use the covering material with a titanium implant by its highly adhesiveness.

### Chapter 2

Carboxyl groups were introduced by ozone exposure to enhance the surface biocompatibility of EVA and high density polyethylene (HDPE). Type I collagen was

immobilized onto the surface through polyioncomplexing. Carboxyl groups on EVA were characterized by ESCA and the neutralization. The amounts of not only the carboxylic group but also collagen increased with increases in time and temperature of ozone exposure. Water-soluble fragments were produced by ozone-exposure to EVA and acted as a collagen crosslinker. Differences in the charge distribution of carboxyl groups affected the amount of the collagen immobilization. Graft polymerization of acrylic acid was also carried out onto EVA and HDPE surfaces. The amount of collagen immobilized by graft polymerization was much higher than that by ozone exposure despite introducing almost the same amounts of carboxylic groups. It was suggested that the negative charge distribution influences the amount of collagen immobilized onto films.

### **Chapter 3**

Human PDL cells were cultured on the EVA surface coated with type I collagen and the cell adhesion and the extension were investigated. Furthermore, collagen type I and fibronectin (FN) syntheses were analyzed. The serum free culture was also tried to investigate the role of collagen in detail.

The results showed that: 1) satisfactory adhesion, extension, and proliferation of the PDL cells on the EVA films coated with collagen were observed, but no good without collagen. 2) immunostaining of cultured PDL cells revealed the syntheses of type I collagen, when cultured on the EVA coated with collagen or conventional culture dish, though FN synthesis was observed even in the EVA without collagen. 3) only PDL cells on the EVA coated with collagen well proliferated in the absence of serum.



## Chapter 4

In this chapter, it was investigated to control the PDL cell differentiation by the surface modification of EVA and to construct the highly organized hybrid implant, and to regenerate the periodontal tissues including cementum, PDL, and alveolar bone. It is thought that these PDL cells have an ability to maintain the structure of the periodontal ligament and the surrounding alveolar bone and the cementum, which suggests that the PDL cells are differentiated to suitable cell phenotypes in the suitable place such as the cementoblasts in the cementum, fibroblasts in the fibrous tissue, and osteoblast in the alveolar bone.

Hydroxyapatite was immobilized on EVA by alternate soaking method following with carboxyl groups introduction through ozone exposure to reveal and control the differentiation of the PDL cells and to develop the highly organized hybrid implant possessing periodontium. Human PDL cells were cultured on the ozone exposed EVA, collagen immobilized EVA, hydroxyapatite immobilized EVA, hydroxyapatite plate, tricalcium phosphate plate, and conventional tissue culture dish. Cell proliferation was the highest on the collagen immobilized EVA and the lowest on the hydroxyapatite immobilized EVA. Alkaline phosphatase activity and osteocalcin secretion were the highest on the hydroxyapatite immobilized EVA. These results suggest that PDL cells were differentiated toward bone like cells on the hydroxyapatite immobilized EVA.

## Chapter 5

In order to investigate the nature of the gingival cells, to control their proliferation and properties and to develop the highly organized hybrid implant possessing periodontium,

hydroxyapatite was immobilized on the EVA by alternate soaking in aqueous  $\text{CaCl}_2$  and  $\text{Na}_2\text{HPO}_4$  solutions, following with carboxyl groups introduction through the ozone exposure. Human gingival cells were cultured on the ozone exposed EVA, collagen immobilized EVA, hydroxyapatite immobilized EVA, and a conventional tissue culture dish. Cell proliferation was the highest on the tissue culture dish and the lowest on the hydroxyapatite immobilized EVA. The results of RT-PCR of gingival cells on the hydroxyapatite immobilized EVA shows that mRNAs expressed in a bone and a periodontal ligament were determined. Furthermore, the alkaline phosphatase activity and ELISA assay revealed that gingival cells acquired the osteoblastic properties when cultured on hydroxyapatite immobilized EVA, suggesting that the periodontium might be regenerated around implant using gingival cells.

## Chapter 6

A trial of PDL around a dental implant was carried out by culturing PDL cells on a EVA coated implant in order to require the functional recovery of the dental implant. The hybrid implant was prepared as follows. The titanium implant was oxidized by hydrogen peroxide. EVA was coated onto the implant and oxidized with ozone gas to introduce carboxyl groups on the surface. The collagen sponge was immobilized around the implant by a freeze-drying method, followed by PDL cell culture onto the implant for 14 days. Then, the cell-seeded implant was applied to the implantation in a canine alveolar bone with a biodegradable membrane that was to prevent a downgrowth of epithelial cells. After 3 months of the implantation, the implants and their surrounding tissue were recovered and the histological analyses were performed. The collagen fibers around the hybrid implant were seemed to be vertically to the alveolar bone similar to those around a natural tooth.

Regenerated PDL were seen in around 30 % of the root part of the hybrid implant, suggesting a possibility of the regeneration of PDL around itself.

## **List of Publications**

### **Chapter 1**

Matsumura K, Hyon S-H, Nakajima N, Peng C, Iwata H and Tsutsumi S.

**Adhesion between poly(ethylene-co-vinyl alcohol)(EVA) and titanium.** J Biomed Mater Res 2002;60:309-315.

### **Chapter 2**

Matsumura K, Hyon S-H, Nakajima N, Peng C and Tsutsumi S.

**Surface modification of poly(ethylene-co-vinyl alcohol)(EVA). Part I . Introduction of carboxyl groups and immobilization of collagen.** J Biomed Mater Res 2000;50:512-517.

### **Chapter 3**

Peng C, Tsutsumi S, Matsumura K, Nakajima N and Hyon S-H.

**Morphologic study and syntheses of type I collagen and fibronectin of human periodontal ligament cells cultured on poly(ethylene-co-vinyl alcohol)(EVA) with collagen immobilization.** J Biomed Mater Res 2001;54:241-246.

### **Chapter 4**

Matsumura K, Hyon S-H, Nakajima N, Iwata H, Watazu A and Tsutsumi S.

**Surface modification of poly(ethylene-co-vinyl alcohol)(EVA) : Hydroxyapatite immobilization and control of periodontal ligament cells differentiation.** Biomaterials 2004; 25:4817-4824.

## Chapter 5

Matsumura K, Hyon S-H, Nakajima N, Iwata H, and Tsutsumi S.

**Differentiation of gingival cells by culturing on hydroxyapatite immobilized poly(ethylene-co-vinyl alcohol)(EVA).** Arch Oral Biol. Submitted.

## Chapter 6

Matsumura K, Hyon S-H, Ikumi N, Nakajima N, Iwata H and Tsutsumi S.

**Periodontal ligament regeneration around hybrid implant; containing inorganic, organic materials and periodontal ligament cell.** in preparation

## Other works

Matsumura K, Hyon S-H, Oka M, Ushio K and Tsutsumi S.

**Scanning electron microscopy and atomic force microscopy observations of surface morphology for articular cartilages of dog's knee and poly(vinyl alcohol) hydrogel.** Kobunshi Ronbunshu 1998;55: 786-790. (in Japanese)

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**Attachment of artificial cartilage to underlying bone.** J Biomed Mater Res PART B 2004; 68B:59-68.

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Institute for Frontier Medical Sciences

Kyoto University

Kazuaki Matsumura

2004